Universidade de Lisboa
Faculdade de Medicina de Lisboa

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Doutoramento em Ciências Biomédicas
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“I don´t feel that is necessary to know exactly what I am. The main interest in life and work is to become someone else that you were not in the beginning”

Michel Foucault

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Summary

Bone metastases are the leading cause of morbidity and mortality among patients with breast cancer. Two different therapeutic approaches are used in patients with advanced metastatic bone disease: anti-tumor and anti-resorptive therapeutics such as bisphosphonates that block osteoclasts activity. Among patients treated with bisphosphonates, around 25% are non-responders. Our studies were based in LMW-PTP, codified by ACP1, a polymorphic enzyme with two main isoforms, called *fast* and *slow*. This protein has been largely associated with cancer, although with contradictory roles. Recently, LMW-PTP has been involved in bone metabolism.

Our first results showed that the LMW-PTP *slow* isoform is more expressed in breast cancer cells compared to non-tumor cells. The *fast* isoform has the opposite pattern. However, both isoforms are involved in migration through a RhoA dependent mechanism, decreasing the migratory potential of cells, confirming previous studies suggesting the importance of GTP/GDP RhoA balance in the migratory potential and not its absolute activity. Suppression of the *slow* isoform in a breast tumor cell line decreased osteoclastogenesis, and this mechanism involved at least Src inactivation and decrease of IL8 production in the tumor cell line. Studies in surgical samples of normal, primary and metastatic breast cancer tissue, confirmed involvement of the *slow* isoform in tumor behavior and showed an increase in the *fast* isoform expression, suggesting that in metastatic tissue, where the vicious cycle of bone metastasis is well established, the *fast* isoform is being regulated by the microenvironment and this microenvironment has the ability to increase the expression of this isoform, possibly as an enhancement mechanism in response to the osteoclastogenic potential of the slow isoform. Regarding therapeutic response to bisphosphonates, there were no differences according to ACP1 genotypes.

Taken together, our results showed that the two main LMW-PTP isoforms may have different roles depending on tumor stage, with the *fast* isoform being more dependent on the tumor microenvironment. Regarding the ACP1 polymorphism, and according to our results, it cannot be used either as a therapeutic response marker or as a prognostic marker.

Sumário

As metástases ósseas são a principal causa de morbilidade e mortalidade entre os pacientes com cancro da mama. São correntemente utilizadas duas abordagens terapêuticas diferentes em pacientes com doença metastática óssea avançada: terapêuticas anti-tumorais e terapêuticas anti-resorptivas, tais como bisfosfonatos, que inibem a actividade osteoclástica. Entre os pacientes tratados com bisfosfonatos, cerca de 25% não respondem à terapêutica. Os nossos estudos basearam-se na LMW-PTP, codificada pelo ACP1, um enzima polimórfico com duas isoformas principais, denominadas fast e slow. Esta proteína tem sido amplamente associada a diferentes tipos de cancro, embora o seu papel permaneça contraditório. Recentemente, a LMW-PTP foi associada ao metabolismo ósseo.

Os nossos primeiros resultados mostraram que a isoforma slow da LMW-PTP é mais expressa em células de cancro da mama do que em células não tumorais. A isoforma fast apresentou padrão de expressão oposto. No entanto, ambas as isoformas estão envolvidas na migração através de um mecanismo dependente do RhoA, diminuindo o potencial migratório das células, e confirmando estudos prévios que sugerem a importância do equilíbrio GTP/GDP RhoA no potencial migratório e não a sua actividade absoluta. A supressão da isoforma slow numa linha celular tumoral da mama diminuiu a osteoclastogénese, e este mecanismo envolve, pelo menos, a inactivação da Src e a diminuição da produção de IL8 pela linha celular. Os estudos realizados em amostras cirúrgicas de tecido normal, primário e metastático de cancro da mama, confirmaram o envolvimento da isoforma slow no comportamento tumoral, e mostraram um aumento da expressão da isoforma fast, sugerindo que no tecido metastático, onde o ciclo vicioso de metastização óssea se encontra bem estabelecido, a isoforma fast está a ser regulada pelo microambiente e este microambiente tem a capacidade de aumentar a expressão desta isoforma, possivelmente como um mecanismo de potenciação em resposta ao potencial osteoclastogénico da isoforma slow. No que diz respeito à resposta terapêutica aos bisfosfonatos, não houve diferenças de acordo com os genótipos do ACP1.

Globalmente, os nossos resultados mostraram que as duas isoformas principais da LMW-PTP podem ter diferentes funções, dependendo do estadio do tumor, com a isoforma fast a mostrar maior dependência do microambiente tumoral. Quanto ao

polimorfismo do *ACP1*, e de acordo com os nossos resultados, este não pode ser usado como um marcador de resposta terapêutica nem como um marcador de prognóstico.

Abbreviations

% - percentage

[1α25$(OH)_2D_3$] – 1α,25-dihydroxyvitamin D_3

ACP1 – Acid phosphatase 1

ADAMTS1 – A disintegrin and metalloproteinase with thrombospondin motif 1

ALP – Alkaline phosphatase

BCCL – Breast Cancer Cell Lines

BMPs – Bone morphogenic proteins

BMU – Basic multicellular unit

BPs – Bisphosphonates

CBFA1 – Core-binding factor 1

CD254 – Cluster of differentiation 254

COX-2 – Ciclo-oxigenase-2

CTIBL - Cancer treatment induced bone loss

CTX – Carboxy [C]-terminal cross-linked telopeptide of type I collagen

CXCL12 – Chemokine (C-X-C) 12

CX_5R – Cysteine – X5- Arginine

CXCR1 receptor – Chemokine (C-X-C) receptor 1

CXCR4 receptor – Chemokine (C-X-C) receptor 4

Cys 12– Cysteine residue in position 12

Cys 17 – Cysteine residue in position 17

Cys-SO – Sulfenic acid

DKK-1 – Dickkopf-1

DMEM – Dulbecco´s Modified Eagle Medium

DNA – Deoxyribonucleic acid

dsPTPs – Dual-specificity PTPs

ECM- Extracellular matrix

EGF – Epidermal growth factors

EMA- European Medicines Agency

EphA2 – Ephrin A2 receptor

Ephrin – Eph family receptor interacting proteins

ERK – extracellular signal-regulated kinase

Fast isoform – LMW-PTP isoform that migrates faster (electrophoretic mobility)

FGF – Fibroblast growth factor

FPP – Farnesyl diphosphate

FPPS - Farnesyl pyrophosphate synthase

GAPs- GTPase-activating proteins

GDP- Guanosine diphosphate

GEFs – Guanosine nucleotide exchange factors

GGPP – Geranyl diphosphate

GTP-Guanosine triphosphate

h-hours

H_2O_2 – Hydrogen peroxide

HMG-CoA – 3-hydroxy-3-methylglutaryl-coenzyme A

ICTP – Type I collagen carboxyterminal telopeptide

IGFs – Insulin growth factor

IgG2- Immunoglobulin G2

IL – Interleukin

Kb- kilobases

k-Ras – Kirstein-Ras-transformed Normal Rat Kidney fibroblasts

LMW-PTPs – Low molecular weight protein tyrosine phosphatases

MAPKs – Mitogen-activated protein kinase

MCF7 – Michigan Cancer Foundation-7 (Breast Cancer Cell Line)

M-CSF – Macrophage colony-stimulating factor

MDA-MB-231- M.D. Anderson – metastatic breast 231 (Breast Cancer Cell Line)

MDA-MB-231 BO – M.D. Anderson – metastatic breast 231 – Clone from bone metastases (Breast Cancer Cell Line)

MDA-MB-231 BO2 – M.D. Anderson – metastatic breast 231 – Clone from bone metastases 2 (Breast Cancer Cell Line)

MDA-MB-435- – M.D. Anderson – metastatic breast 435 (Breast Cancer Cell Line)

mg- miligrams

ml- mililiter

mmol - milimol

MMPs – Metalloproteinases

MMP1- Metalloproteinase 1

MMP8 – Metalloproteinase 8

MMP9- Metalloproteinase 9

MMP13 – Metalloproteinase 13

mRNA – Message ribonucleic acid

NaCl – Sodium cloride

NFATc1 – Nuclear factor of activated T-cells, cytoplasmic 1

NF-kB – Factor nuclear kappa B

NIH3T3 – National Institute of Health – 3 days transfer (Mouse embryonic fibroblasts)

nmol – nanomol

NO – Nitric Oxide

Nox-1 – NADPH oxidase 1

nrPTPs – non-receptor protein tyrosine phosphatases

NTX – amino [N]-terminal cross-linked telopeptide of type I collagen

ODF – osteoclast differentiation factor

OPG – osteoprotegerin

OPGL – osteoprotegerin ligand

p190RhoGAP – p190 Rho GTPase activating protein

PC3 – Prostate Cancer 3 (Prostate Cancer Cell Line)

PDGF – platelet derived growth factors

PDGF-R – Platelet Derived Growth Factor Receptor

PGE2 – prostaglandin E2

PTH – parathyroid hormone

PTH-rp –parathyroid hormone-related peptide

PTKs – protein tyrosine kinases

PTPN23 – protein tyrosine phosphatase 23

PTPs – protein tyrosine phosphatase

pTyr – phosphorylated tyrosine

RANK – Receptor activator of nuclear factor kappa-B

RANKL – Receptor activator of nuclear factor kappa-B ligand

RAW 264.7 – Mouse leukaemic monocyte macrophage cell line

RhoGDIs – RhoGTPases dissociation inhibitors

RNA – ribonucleic acid

RNAi – ribonucleic acid interference

ROS – reactive oxygen species

RPTPs – receptor protein tyrosine phosphatases

Runx-2 – Runt-related transcription factor 2

siRNA- small-interference ribonucleic acid

Slow isoform – LMW-PTP isoform that migrates slow in electrophoresis (electrophoretic mobility)

Src – Proto-oncogene tyrosine-protein kinase Src

SREs- Skeletal related events

TGFα – Transforming growth factor α

TGFβ – Transforming growth factor β

TIMP-2 – Tissue inhibitor of metalloproteinases 2

TNFSF11- Tumor necrosis factor ligand superfamily member 11

TNF-α – Tumor necrosis factor-α

TR- Tranfection Reagent

TRAF6 – Tumor necrosis factor receptor-associated factor 6

TRAIL – TNF-related apoptosis inducing ligand

TRANCE – TNF-related activation-induced cytokine

TRAP- Tartarate Resistant Acid Phosphatase

Try527 – Tyrosine residue in position 527

Tyr416 – Tyrosine residue in position 416

ZR-75 – Human Breast Carcinoma cell line

Overview of the thesis

Over the years preceding the execution of this work, compelling evidence had indicated an essential involvement of LMW-PTP in the regulation of important processes associated with carcinogenesis. Given the lack of studies concerning the role of the two main LMW-PTP isoforms in this process, we proposed to study how these isoforms could be players in carcinogenesis. Furthermore, all our approaches had the main aim of clarifying how the differential expression of the two LMW-PTP isoforms could influence bone metastasis patients' response to anti-resorptive drugs.

Besides breast cancer, there are other cancers that frequently metastasize to bone, namely prostate cancer. However, in this thesis, and due to the high prevalence of breast cancer patients in the Oncology Department from Hospital de Santa Maria and the availability of breast cancer cell lines, we used breast cancer as a model for the study of bone metastization.

The thesis is organized in five chapters. Chapter 1, a general introduction, describes the mechanisms of bone metastases in the context of breast cancer. Also in chapter 1 the interplay between bone and LWM-PTP isoforms is explored, as well as the biological functions of these proteins.

Chapters 2, 3, 4, 5, 6 present the results obtained in the context of this thesis, and one of them was published as scientific papers. Chapter 7 is a published review paper focusing LMW-PTP in oncogenesis. The global approach of each chapter is summarized in Figure 0.1.

Chapter 8 is a final discussion of key findings obtained throughout this thesis and the putative relevance of the new mechanisms described for the LMW-PTP isoforms in the context of carcinogenesis and bone metastases.

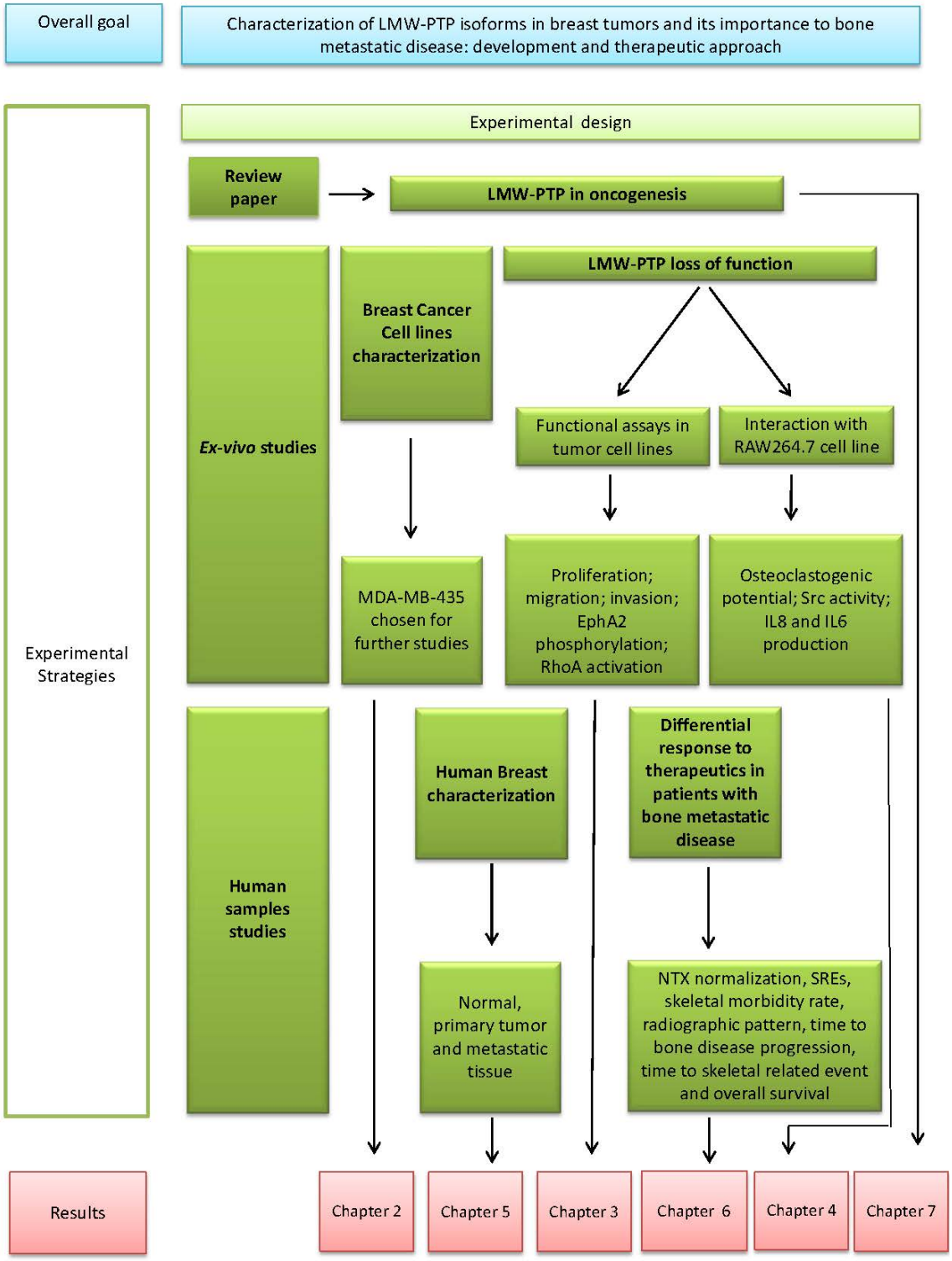

Figure 0.1 Conceptual framework used for the investigation of LMW-PTP and its two main isoforms, *fast* and *slow*, in tumor progression and bone metastases. Organization of the obtained results.

Chapter 1

Introduction

1 Introduction

1.1 Breast cancer – statistics

Breast cancer is by far the most frequent cancer among women, with an estimated 1.38 million new cancer cases diagnosed in 2008 (23% of all cancers), and ranks second overall (10.9% of all cancers). It is now the most common cancer both in developed and developing regions, with around 690,000 new cases per year estimated in each region (1).

Incidence rates vary from 19.3 per 100,000 women in Eastern Africa to 89.7 per 100,000 women in Western Europe, and are high (greater than 80 per 100,000) in developed regions (except Japan) and low (less than 40 per 100,000) in most of the developing regions (1).

The range of mortality rates is much lower (approximately 6-19 per 100,000) due to the more favorable survival of breast cancer in high-incidence developed regions. As a result, breast cancer ranks as the fifth cause of death from cancer overall (458,000 deaths per year), but it is still the most frequent cause of cancer deaths per year in women in both developing (269,000 deaths, 12.7% of total) and developed regions, where the estimated 189,000 deaths is almost equal to the estimated number of deaths from lung cancer (188,000 deaths).

In Portugal, 4,500 new cases of breast cancer are diagnosed every year and 1,500 patients die every year with breast cancer.

Approximately 90% of breast cancers are curable if detected in an early phase of the disease (2).

1.2 Cancer and metastases – Interactions Between Tumor And Host

Cancer is the general term used for diseases in which cells become abnormal and divide without control and are able to invade other tissues. Cancer cells may invade surrounding tissues and may spread through the bloodstream and lymphatic system, colonizing different parts of the body. The hallmarks of cancer comprise six biological abilities during the multistep development of human cancers (3). These are distinct and

complementary capabilities that enable tumor growth and metastatic dissemination, providing a logical framework for understanding the remarkable diversity of neoplastic diseases. These hallmarks are: 1) sustaining proliferative signaling, 2) evading growth suppressors, 3) activating invasion and metastases, 4) enabling replicative immortality, 5) inducing angiogenesis and 6) resisting cell death (4). Gain-of-function mutations of proto-oncogenes or loss-of-function mutations of tumor suppressor genes underlie excessive cell division, migration and invasion (5).

Breast cancer is the most common malignancy and second major cause of morbility and mortality in Western women (6). If detected at an early-stage the prognosis is favorable, with a five-year survival rate for death from the cancer of 98%. However, when the initial diagnosis is of advanced metastatic disease, the five-year survival rate decreases to 26% (7). The leading cause of mortality among these patients is metastases at distant sites.

Approximately 10-15% of patients with breast cancer have an aggressive disease and develop metastases within 3 years after initial detection of the primary tumor, although metastases can occur at any moment during the course of the disease (6).

Metastasis is the systemic dissemination of tumor cells at sites distinct from the primary lesion. It is a multistep process that involves detachment of cells from the primary tumor, followed by survival in the blood vessels or lymphatic systems and finally development of a secondary tumor (8). This process is regulated not only by changes in tumor cells but also by interactions with the surrounding microenvironment (6). The first evidence that metastases formation depends on intrinsic characteristics of the tumor cells was derived from serial passage of a melanoma cell line through mice, which resulted in sub-lines with increasing invasive potential (9).

The risk of metastases' development increases with the presence of lymph-node metastases, a larger-sized primary tumor and loss of histopathological differentiation grade. These markers are considered well established prognostic markers (6). However, these traditional prognostic markers are only able to confidently identify a group of 30% of patients.

Why cancer cells metastasize is one of the most important issues in tumor biology. The understanding of molecular mechanisms of the metastatic process will undoubtedly improve clinical management of the disease. According to the widely accepted model of metastases, or traditional model (6), rare subpopulations of cells within the primary

tumor acquire advantageous genetic alterations over time, which enable them to metastasize and form solid tumors at distant sites (10). However, as research in this field progresses, different models of the metastatic cascade have been proposed: 1) Spontaneous metastases assays indicate that all tumor cells have the ability to develop metastases; 2) the Dynamic heterogeneity model proposes that the frequency with which metastatic variants arise within the primary tumor determines its metastatic potential – metastatic subpopulations are generated at high rates in a primary tumor, but these variants are relatively unstable, resulting in a dynamic equilibrium between generation and loss of metastatic variants; 3) the Clonal dominancy theory proposes that metastatic subclones within a primary tumor can overgrow and dominate the tumor mass itself; and 4) the Genometastases hypothesis proposes that metastasis occurs through transfection of susceptible cells in distant organs with circulating oncogenes derived from the primary tumor (6). Based on these models, and integrating the knowledge of novel prognostic markers and gene expression profiles, a new model of metastases has been proposed – the integrative model of breast cancer metastases (6). In this model, primary breast carcinomas with metastatic potential can be distinguished from those that have a low likelihood of metastases by their gene expression profiles – the poor- and good-prognosis signatures, respectively, determined by the 70-gene expression profile (11). Many genes have been identified whose increased expression correlates with metastases. Indeed, primary tumors may already contain a gene expression profile that is strongly predictive of metastases and poor survival, challenging the notion that metastatic ability is acquired later during tumor progression (11, 12).

This model proposes that oncogenic mutations occurring in a breast stem cell can cause its transformation to a breast cancer stem cell, generating “poor-prognosis” tumors. Mutations occurring in differentiated progenitor cells might form a non-metastatic “good-prognosis” breast carcinoma. In the metastatic poor prognosis tumors, under the influence of stromal fibroblasts, only the population of breast cancer stem cells has the ability to metastasize. In the metastasis site, the disseminated cancer stem cells would again induce a similar stromal response as in the primary breast tumor (6).

Metastases genes encode homing receptors, their ligands, associated signaling molecules, and extracellular matrix-degrading proteases, which jointly cause invasion and anchorage-independent survival. The organ preference of metastasis formation is

determined by the specific identity of the homing receptors expressed on the tumor cell surface and their cognate cytokine ligands (5).

The genetic background from which cancer arises also has an effect on the ability of mouse mammary tumor cells to metastasize, suggesting that the propensity to metastasize is, in part, influenced by the normal genetic background of the host (13). Thus, the outcome of metastases depends on multiple interactions ("cross-talk") of metastasizing cells with homeostatic mechanisms. (14).

This tumor-host crosstalk supports the hypothesis that co-targeting cancer cells and tumor stromal cells can be a viable approach for mammary cancer prevention and treatment (8).

In his "seed and soil" hypothesis, Paget proposed that tumor cells "seeds" can colonize microenvironments, or "soils", that are compatible with their growths (15). According to this theory, the main steps in the formation of metastases are: **a)** cellular transformation and tumor growth; **b)** primary tumor proliferates and angiogenesis occurs; **c)** primary tumor cells detach from the initial location and invade lymphatic vessels, venules and capillaries; **d)** embolization of single tumor cells or aggregates and the survivor tumor cells become trapped in the capillary beds of distant organs; **e)** extravasation and establishment of a microenvironment; extravasation is facilitated by cancer cell secretion of matrix metalloproteinases (MMPs) and cathepsin K that destroy the surrounding tissue (16); **f)** proliferation within the organ parenchyma completes the metastatic process only if there is an appropriate environment of paracrine or autocrine factors that aid in growth and vascularization (14, 16).

Once disseminated, metastases from breast carcinoma emerge in various organs, although the most common sites for breast cancer metastases are bone, lung and liver (17). The distribution pattern of cancer cells to the bone is believed to be due to the venous flow from breast cancer towards the vena cava and into the vertebral venous plexus. Once in circulation, entry of the cancer cells into the venous circulation of the bone marrow may be facilitated by the slow blood flow and particular anatomy of the venous sinusoids. Nonetheless, these steps alone do not explain survival and growth of cancer cells in bone (16). The preference of breast cancer cells for bone as a metastatic site is confirmed by the fact that 65-75% of patients with advanced disease develop bone metastases (18).

Bone metastases often cause important bone complications (19) – skeletal related events (SREs) - associated with bone metastases, and these include: hypercalcemia, pathologic fracture, radiation to bone, spinal cord compression, and surgery to bone, all resulting in a significant decrease of the patient´s quality of life, and increasing morbility and mortality (20). The incidence of these complications in breast cancer patients with bone metastases without bone-targeted therapy is 64% (21). Bone metastases are also the most frequent cause of pain in cancer patients (22) Once tumors metastasize to bone, they are irreversible: after diagnosis of bone metastases, the five-year survival rate of patients with breast cancer is 20% (23).

Not all bones of the skeleton are equally favoured for metastases: spinal vertebrae, ribs, and the ends of long bones are preferred sites of metastases. In general, well vascularized areas and areas of the skeleton containing red marrow are the sites of metastatic colonization (16).

During the process of bone metastization, tumor cells use bone microenvironment and the factors released by bone cells, such as osteoblasts and osteoclasts, to survive and proliferate. Therefore, to better understand bone metastization, it is important to clarify bone physiology.

1.3 Bone physiology

Bone is a unique microenvironment and a specialized connective tissue. It provides structural support, has protective functions and plays a major role in the regulation of calcium levels (16). Bone has a calcified extracellular matrix in which cells are embedded: osteoblasts, osteoclasts and osteocytes.

Osteoblasts are mononuclear, non terminally differentiated, specialized mesenchymal cells (24). A transcription factor that is critical for osteoblasts differentiation is Runx-2, or core-binding factor 1 (CBFA1). CBFA1 drives the expression of most genes associated with osteoblast differentiation. The differentiation of osteoblasts is less well understood than the differentiation of osteoclasts, but it is clear that there is an early osteoblast precursor that produces alkaline phosphatase, and a more differentiated precursor that produces increasing amounts of osteocalcin and calcified matrix (23)

After synthesizing new bone matrix, the osteoblast either undergoes apoptosis or becomes embedded in the bone as an osteocyte (16). Osteocytes are differentiated

osteoblasts entrapped in the lacunae. Osteocytes are the most abundant cells in bone, communicating with the other cells and the surrounding medium through extensions of their plasma membrane (24).

Osteoclasts are the cells that resorb bone and are differentiated from hematopoietic cells with a monocyte/macrophage lineage. Mature osteoclasts are giant (50 to 100μm diameter) and multinucleated cells (25) with abundant mitochondria, numerous lysosomes, and free ribosomes.

Osteoblasts produce the osteoclast differentiation factor receptor activator of NF-κB ligand (RANKL) (identical to TRANCE, ODF, OPGL, TNFSF11, and CD254) in response to several bone resorbing factors, such as 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$] and parathyroid hormone (PTH) (26, 27). On osteoclasts precursors, binding of RANKL to its receptor RANK in the presence of M-CSF (Macrophage-colony stimulating factor), promotes cellular fusion of several monocytes to form a multinucleated osteoclast that results in recruitment of TNF receptor-associated factor (TRAF) family proteins such as TRAF6, which activate NF-κB and MAP kinases (MAPKs). Such signaling subsequently activates the transcription factors c-fos, PU.1, and NFATc1, all of which are required for osteoclast differentiation. Among these factors, NFATc1 is selectively induced by stimulation with RANKL and functions as a master switch for regulating terminal differentiation of osteoclasts (28). Disruption of RANKL or RANK results in osteopetrosis due to impaired osteoclast differentiation (29), indicating that the RANK-RANKL system is essential for the regulation of osteoclast differentiation in vivo. Osteoprotegerin (OPG), a secreted disulfide-linked dimeric glycoprotein, is another molecule important for osteoclastogenesis. This protein has very potent inhibitory effects on osteoclastogenesis and bone resorption. The antiosteoclastogenic property of OPG is due to its ability to act as a decoy by binding to RANKL and blocking the RANKL/RANK interaction (25).

M-CSF participates in the later differentiation stage through activation of Akt, c-Fos and ERK (extracellular signal-regulated kinase) pathways, although it seems to be mainly important in an early phase of osteoclastogenesis, inducing the proliferation of osteoclast precursor cells, supporting their survival and upregulating RANK expression, which is a prerequisite for osteoclast precursor cells (27). Therefore, osteoclast differentiation is supported by osteoblasts as well as stromal cells through cell-to-cell interactions (30).

Activated osteoclasts bind to the bone matrix through $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_2\beta_1$ integrins located on the membrane surface and also secrete acid and lysosomal enzymes which degrade bone (16). After the osteoclast adhesion to bone matrix, the formation of the ruffled border depends on tyrosine kinase Src (31). The ruffled border is a typical morphological feature of osteoclasts, that is a complex system of finger-shaped projections of the membrane, whose function is to mediate the resorption of the calcified bone matrix (32). The ruffled border is completely surrounded by a clear zone – the cytoplasm in the clear zone has a uniform appearance and contains bundles of actin-filaments. The clear zone delimits the area of attachment of the osteoclast to the bone surface and seals off a distinct area of the bone surface that will be excavated. The ability of the clear zone to seal off this area of the bone surface allows the formation of a microenvironment suitable for the operation of the resorptive apparatus (25). Other types of proteins, small GTPases, have a crucial role in osteoclastic activity. Small GTPases localize to specific membrane compartments. This property is dependent on post-translational prenylation, which involves the attachment of a hydrophobic isoprenoid lipig group (a 15-carbon farnesil or a 20-carbon geranyl-geranyl moiety) to a conserved cysteine residue contained within characteristic prenylation motifs in the C-terminus of small GTPases, such as Rho. Rho was the first GTPase to be studied in osteoclasts and different studies show that RhoA activity is essential for osteoclast polarization (33). During osteoclastic bone resorption, the acidic environment created by the ATP-driven proton pump of the resorption site, dissolves the mineral component of the matrix. Matrix components, mainly collagen, are degraded by matrix metalloproteinases (MMPs) and cathepsins K, B, and L, secreted by the osteoclast into the area of bone resorption (25). TRAP (tartarate resistant acid phosphatase) is another feature of osteoclasts: this enzyme is commonly used for the detection of osteoclasts in bone specimens (23).

Bone is constantly being remodeled by a process in which osteoblasts are responsible for bone formation and osteoclasts for bone resorption. Bone resorption and bone formation, however, are not separate, independently regulated processes. In the adult skeleton, all osteoclasts and osteoblasts belong to a unique temporary structure, known as basic multicellular unit or BMU (34). BMU, which is approximately 1-2 mm long and 0.2-0.4 nm wide, comprises an anterior group team of osteoclasts, a posterior group of osteoblasts, a central vascular capillary, a nerve supply, and associated connective

tissue (34).The extracellular matrix is hard-mineralized by the deposition of hydroxyapatite. This hard-mineralized matrix stores abundant varieties of growth factors such as insulin growth factor (IGF), transforming growth factor α and β (TGFα and β), fibroblast growth factors (FGF-1 and -2), platelet derived growth factors (PDGFs) and bone morphogenic proteins (BMPs). The majority of these growth factors are released in their active form into the marrow when bone is degraded by osteoclasts. They could provide a fertile ground, which is an attractive site for metastastic cancer cells (16), since their chemotactic properties allow tumor cells to grow (35).

As BMU advances, osteoclasts leave the resorption site and osteoblasts move in to cover the excavated area and begin the process of new bone formation by secreting osteoid, which is eventually mineralized into new bone (25).

1.4 Bone metastases: osteolytic and osteoblastic

Bone metastases from breast cancer are a good example of interaction between tumor cells and host, in this case, bone cells. The success of bone metastases depends on a complex crosstalk between tumor cells and bone microenvironment (36).

Based on the radiographic pattern of bone lesions (37), bone metastases can be divided into two broad categories: osteolytic metastases, associated with bone destruction, and osteoblastic metastases, associated with new bone formation. Although activation of osteoclasts is a common feature in both osteolytic and osteoblastic bone metastases, the development of either type of lesion depends on osteoblasts (31): inhibition of osteoblasts results in osteolysis and the increase in osteoblasts activity results in osteoblastic lesions (23). Most breast cancer metastases are associated with osteolytic lesions, but up to 15% are osteoblastic or mixed (36).

Cancers such as prostate cancer tend to be predominantly osteoblastic in nature. Excess bone deposition occurs but not necessarily in an ordered fashion (16). In breast cancer, endothelin-1 is associated with bone osteoblastic lesions, stimulating the formation of new bone through osteoblast proliferation, and serum endothelin-1 has been found to be increased in patients with osteoblastic prostate cancer metastases (38, 39).

Regardless of tumor type, patients with bone metastases have evidence of both abnormal bone resorption and formation. Autopsy results show that bone metastases are phenotypically heterogeneous (40) within and between lesions but, despite this heterogeneity, multiple metastatic foci seem to be monoclonal (41).

The "vicious cycle" hypothesis (Figure 1.1) describes how tumor cells interact with the bone microenvironment to drive bone destruction and tumor growth in a symbiotic relationship (19). The time a primary tumor cell takes to disseminate and colonize distant organs is very variable: tumor cells can lie dormant in the bone marrow in humans for several months or years before receiving appropriate signals to proliferate sufficiently to induce osteolytic lesions. For instance, breast cancer cells can induce osteolytic lesions 15-20 years after excision of the primary tumor (42).

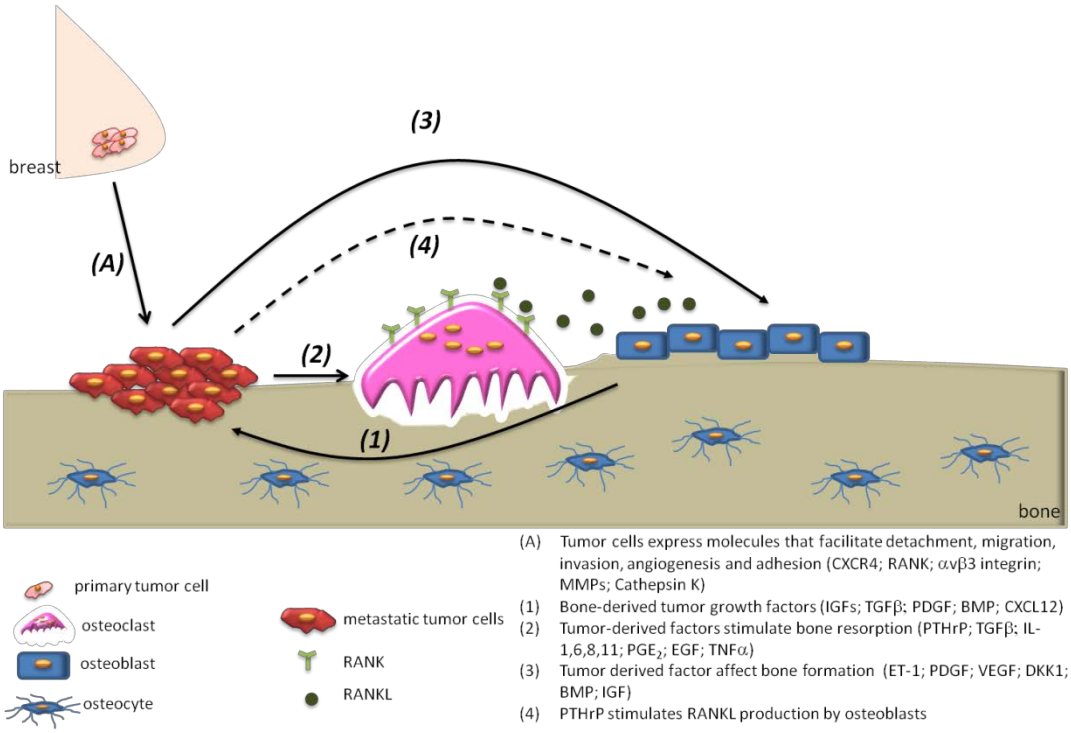

Figure 1.1– Lytic bone metastasis formation in breast cancer. Several steps in the activation and proliferation of osteoclasts and also the interaction between tumor cells and bone microenvironment are represented. The vicious cycle of bone metastases, through which osteoclastic bone resorption cause release of growth factors which can act on breast cancer cells, is shown.

The anchoring of breast cancer cells to the bone microenvironment disrupts the normal regulatory communications between osteoblasts and osteoclasts, favoring the balance towards increased osteoclast activity, resulting in excessive bone resorption (43). Breast cancer cells cannot directly resorb bone (at least mineralized bone), and must exert this effect indirectly through the action of osteoclasts (44). It seems that osteoblasts are

important mediators of breast tumor cell-induced osteoclastogenesis – breast cancer cells stimulate immature osteoblasts to produce a variety of factors, including RANKL, prostaglandin E and IL-11, which in turn stimulate osteoclast differentiation from monocyte precursors (45). On the other hand, the resorption of bone matrix leads to the release of factors that stimulate tumor growth ("vicious cycle") (36). The factors surrounding tumor cells not only support their growth but also alter their phenotype, rendering them resistant to standard cytotoxic anti-tumor treatments (46, 47).

Breast cancer cells induce formation of osteoclasts by secreting osteotropic cytokines such as parathyroid hormone-related peptide (PTH-rP), tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2), interleukins (IL-1, IL-6, IL-8, IL-11, IL-15 and IL-17) (35) and MMPs (48). PTH-rP is thought to control the proliferation/differentiation that is linked to the compensatory growth of the mammary gland during lactation, being overproduced in about 60% of primary breast cancers (35, 49).PTH-rP is an osteoclast-activating factor, exerting its effects by binding to receptors present on osteoblasts, which in response secrete factors such as RANKL and M-CSF resulting in osteoclast activation and bone resorption.

TGF-β is stored within the bone matrix and is released during osteoclastic bone resorption, being a crucial cytokine for the development and progression of bone metastases. It is one of the main components of a paracrine loop that can be responsible for the affinity with which breast cancer cells colonize bone (35). TGF-β binds to a heterodimeric receptor and activates the canonical Smad-dependent and Smad-independent signaling pathways, stimulating PTH-rP production by breast cancer cells growing in bone (35, 36). In an advanced stage, cancer cells often escape growth inhibition by TGF-β, which instead can activate epithelial-mesenchymal transition and invasion, promoting metastases. TGF-β also increases angiogenesis and suppresses immune surveillance of tumor cells (36).

COX-2 levels and prostaglandin 2 (PGE_2) have also been found to contribute to osteoclast activation and facilitate the establishment of a microenvironment for cancer cell metastasis. COX-2 levels and activity correlate with cancer cell metastases (16).

The involvement on MMPs and related metalloproteinases in bone metastases has been speculated due to the bone remodeling defect of MMP-deficient animals, the production of MMPs by osteoclasts during bone resorption and the ability of MMPs to degrade major components of bone matrix (48). The functional role of MMP1 and ADAMTS1

in promoting osteolytic metastases in explained by Lu X et al: these two proteinases acts in concert not only to enhance invasion through the ECM and endothelium, but also to promote tumor colonization in the bone microenvironment through a pro-osteolytic signaling cascade. This cascade promotes osteoclast differentiation by shedding tumor-derived epidermal growth factor (EGF)-like factors decreasing osteoblast production of OPG (osteoprotegerin) in the local of bone microenvironment (48).

The Interleukine 6 family has been discovered around 25 years ago, and since then its effects are still being unraveled. Is has been originally described as a macrophage differentiation factor, but soon IL-6 appeared to be produced by stromal/osteoblastic cells and to enhance osteoclast formation in various culture systems. IL-6 type cytokines can stimulate osteoclasts differentiation and bone resorption by an indirect mechanism, increasing interactions between osteoblasts and osteoclasts (50). The pro-tumoral effects of IL-6 type cytokines are related to their ability to enhance tumor cell proliferation, cell motility, invasion and angiogenesis and has been implicated in tumor growth in bone – cancer cells that produce high levels of IL-6 induce bone resorption. Considering that bone resorption leads to the release of several bone-associated growth factors, this effect is presumably relevant to refill the vicious cycle between bone resorption and tumor proliferation (50).

Breast cancer cells isolated from bone metastases express IL-8, a molecule that supports the maturation of osteoclasts and cause increased levels of bone metastases in model systems (42). IL-8 is produced by a variety of cell types such as macrophages, neutrophils and endothelial cells in response to inflammation and injury, and is produced by a variety of tumor cell types. IL-8 is, therefore, being implicated in tumor progression via its ability to enhance angiogenesis, cell motility, and invasion, which appears to correlate with the metastatic potential (51). Elevated levels of IL-8 in serum of breast cancer patients predict early metastatic spread, and human breast cancer cell lines expressing IL-8 induce high levels of bone metastases compared to IL-8 negative cell lines (42). This clinical correlation with both tumor aggressiveness and patient survival supports an important contribution of IL-8 to osteolytic metastases. Bendre et al reported that IL-8 stimulated both osteoclastogenesis, through binding the CXCR1 receptor present on osteoclasts and their precursors and, consequently bone resorption. They also showed that IL-8 was able to regulate the expression of RANKL by osteoblastic stromal cells, implicating IL-8 as a potent activator of the bone destruction

common in metastatic bone disease (51). The mechanism by which tumor derived interleukin-8 stimulates osteoclastogenesis is independent of RANKL (52).

The Wnt (16) signaling pathway also plays a role in both osteogenesis and oncogenesis (36). Wnt proteins released by metastatic prostate cancer stimulate osteoblasts and have autocrine effects on tumor proliferation (53). An inhibitor of Wnt signaling, dickkopf-1 (DKK-1), can regulate metastatic progression by opposing osteogenic Wnts early in metastases, and controlling the phenotypic switch from osteolytic to osteoblastic lesions later in the metastatic process (54). Elevated DKK-1 expression is an early event in prostate cancer, declining in advanced bone metastases. The decrease of DKK-1 in bone metastases can relieve Wnt inhibition, increasing osteoblast activity. These results suggest that the involvement of DKK-1 dictates whether bone metastases are osteoblastic or osteolytic, emphasizing once more the importance of the bone microenvironment (16).

The proto-oncogene Src, encoded by the *C-SRC* gene, is a non-receptor membrane-associated tyrosine kinase that belongs to the family of Src kinases. Upon activation, this kinase is involved in cellular proliferation, survival, migration and angiogenesis – four important steps in cancer development (55). Although Src is widely expressed, the majority of cell types display low levels of it, while mature osteoclasts, for instance, express high levels (56). In osteoclasts, Src is activated following integrin binding after these cells attach to bone matrix to start the resorptive process and also after RANKL binding to RANK. The pathway involving Src is a key pathway during normal, healthy bone turnover and seems to be essential for the normal organization of osteoclasts' cytoskeleton (57). Src is essential for osteoclast activation and osteoblast inhibition. During tumor progression, Src activates pathological processes thereby supporting tumor growth, metastization and tissue invasion (56). Furthermore, increased expression and activity of Src have been described in a wide variety of tumor types, eg prostate, colon and breast (58). In breast and prostate cancer, Src assumes a more important role due to the ability of these tumors to metastasize to bone. Increased Src activity is known to correlate with tumor progression, with the metastatic tissue showing the highest activity. Pre-clinical studies demonstrated that decreased Src expression enhances osteoblast proliferation and bone formation (59). Different Src inhibitors have been developed, and several of them are currently being used in clinical trials (56). The most well known Src inhibitors are Bosutinib (SKI-606), Saracatinib (AZD0530) and

Dasatinib (BMS354825). Bosutinib is under phase I and II Clinical Trials in solid tumors and in phase III in hematologic malignancies. Saracatinib is under phase I and II as monotheraphy and in combination in multiple solid malignancies. Finally, Dasatinib is in phase I and II as monotherapy and in combination (solid tumors); there are biomarkers selected trials under phase II trials in hematologic malignancies (60).

It is clear that the organ microenvironment has a crucial influence in cancer cell spreading to a specific location. Crosstalk between the cancer cell "seed" and the target organ microenvironment "soil" will determine if the cancer cell metastasizes to a specific site and if that microenvironment supports growth and proliferation of the metastatic cancer cell. Bone provides an especially attractive site for a variety of reasons. Metabolic active areas of bone are well-vascularized, having a vascular/capillary network that allows various cells to easily enter and exit. The normal remodeling process provides chemotactic and growth factors that attract cancer cells and support them once in place. The bone matrix contains a rich storehouse of growth factors such as TGF-β that are released during bone turnover. Resident cells thrive in the rich cocktail of released cytokines. Finally, both osteoblasts and osteoclasts activities can be modulated by cancer cells to their advantage. The release of characteristic sets of cytokines by the bone matrix of an osteolytic or osteoblastic lesion will facilitate the chemoattraction and survival of metastatic cancer cells. Understanding the mechanisms underlying these events will allow the development of novel therapeutic approaches that specifically target metastases and manipulate the organ microenvironment (16).

1.5 Bone metastases therapies

Current management of bone metastases is aimed primarily at reducing morbidity due to SREs so that quality of life and functional independence can be preserved or improved. Therefore, the management goals of metastatic disease to bone are to maximize pain control, achieve functional preservation and restoration, to stabilize the skeleton and to control the tumor locally (61). Current therapies of bone metastases include pain management/analgesia, systemic therapy (bone cell targeting agents, chemotherapy, hormone therapy), radiation therapy (external-beam radiation therapy,

radiopharmaceuticals), surgery to correct fractures or spinal cord compression, and bone-target agents (such as bisphosphonates or denosumab) to prevent bone complications and to additionally treat bone pain.

The stimulation of osteoclast function by tumor cells is of particular importance: it results in osteolysis, which is typically associated with disruption of the normal coupling signals between osteoblast and osteoclast function, and is the rationale for the use of anti-resortive drugs such as bisphosphonates or denosumab in the management of metastatic bone disease (62).

1.5.1 Bisphoshonates

Targeting the osteoclast has become a standard therapeutic approach for metastatic bone disease. Clinically, patients with breast cancer and other osteolytic metastases, as well as osteoblastic prostate bone metastases, are treated with bisphosphonates that inhibit osteoclast activity. Bisphosphonates are extensively used in the management of patients with bone metastases with the aim of preserving the patients´ functional independence and quality of life, by preventing and delaying SREs, thereby controlling bone pain and reducing the need for analgesics and palliative radiotherapy (63-65).

Bisphosphonates predominantly localize to bone due to the high affinity of the bisphosphonate moiety to calcium. Studies in animals have shown that bisphosphonates are primarily deposited in newly formed bone and underneath osteoclasts.

Bisphosphonates are stable analogues of pyrophosphate, used in pathologies characterized by excess bone resorption. These drugs have high affinity for the hydroxyapatite phase of the bone matrix. They cause cellular toxicity with low specificity, metabolized to non-hydrolyzable ATP analogues (first generation bisphosphonates such as atidronate, tiludronate and clodronate) or, in the case of nitrogen-containing bisphosphonates (pamidronate and zoledronate) inhibit farnesyl pyrophosphate synthase (FPPS) from the mevalonate pathway (66) (Figure 1.2). FPPS inhibition causes loss of farnesyl- and geranyl-phosphates, required for prenylation of signaling GTPases, such as Ras, Rho and Rac – essential for cell function and survival (67). As a consequence, defective intracellular vesicle transport and loss of prenylated proteins occurs, leading to osteoclast apoptosis via activation of the caspases´ cascade

(68). The accumulation of isopenthyl diphosphate will cause loss of mitochondrial membrane potential and direct apoptosis induction (69) (Figure 1.2).

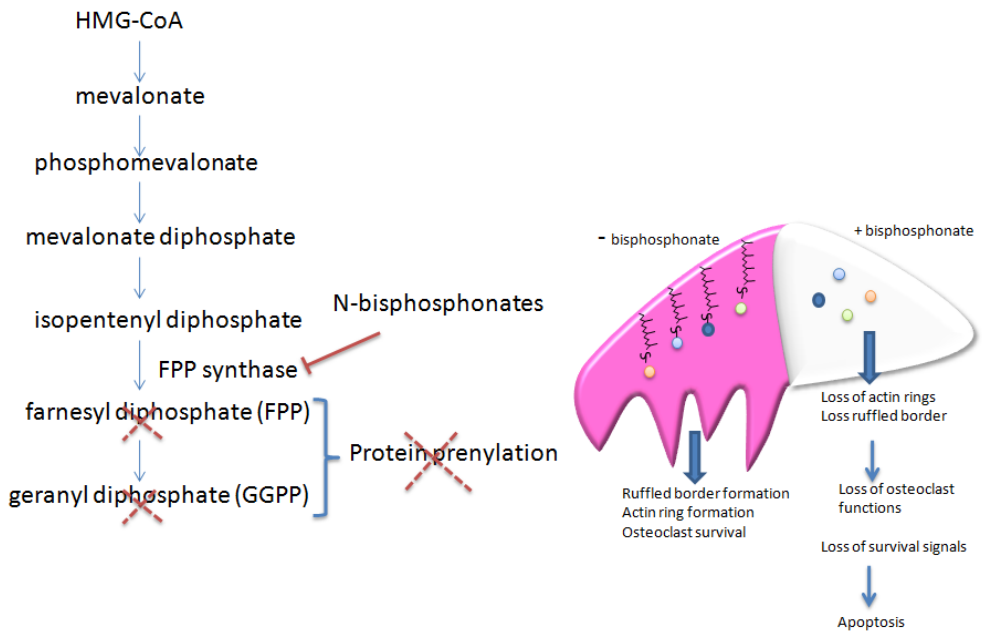

Figure 1.2 - Schematic representation of the mevalonate pathway (left panel). Nitrogen-containing bisphosphonates inhibit FPP synthase, thereby preventing the synthesis of FPP and GGPP required for protein prenylation. Inhibition of protein prenylation disrupts specialized features of the osteoclast required for bone resorption (right panel). Adapted from Rogers M. (2003) (70).

Clodronate, ibandronate, pamidronate and zoledronate are currently approved by EMA for bone metastases (Table 1.1). Clodronate is associated with both decreased bone metastases and death rate in patients with breast cancer (71). Ibandronate reduced skeletal morbidity and significantly delayed the time to first SRE in patients with breast cancer and bone metastases (72).

Table 1.1 – Bisphosphonates approved for use in different types of cancer

	Indication				
	Prevention of SREs				
	HCM	Multiple Myeloma	Breast Cancer	Prostate Cancer[a]	Other Solid Tumors
Clodronate (Oral)[b,c]	✓	✓	✓		
Pamidronate (IV)[b,d]	✓✓	✓✓	✓✓		
Zoledronic Acid (IV)[b,e]	✓✓	✓✓	✓✓	✓✓	✓✓
Ibandronate (Oral)[b,f]			✓		
Ibandronate (IV)[b,g]	✓		✓		

HCM = hypercalcemia of malignancy; IV = intravenous; SRE = skeletal-related event

✓ - European Registration ✓- US Registration [a] In the United States, prostate cancer must have progressed despite hormone therapy. b. Bonefos [package insert]. Toronto, Canada, Bayer Inc, 2010; c. Loron (tablets). Summary of Product Characteristics, Welwyn Garden City, UK, Roche, 2008; d. Aredia [package insert]. Novartis Pharmaceuticals; East Hanover, NJ, 2008; e. Zometa [package insert]. Novartis Pharmaceuticals; East Hanover, NJ, 2009; f. Bondronat (tablets). Summary of Product Characteristics, Horsholm, Denmark, Apotex Europe BV, 2010; g. Bondronat (vials). Summary of Product Characteristics, Welwyn Garden City, UK, Roche, 2010;

Of the currently available bisphosphonates, zoledronic acid (zoledronate) and pamidronate, both nitrogen-containing bisphosphonates, are the most potent inhibitors of bone resorption *in vitro* and *in vivo*, and have also been reported to induce apoptosis of tumor cells *in vitro* (73). These drugs acts primarily on mature osteoclasts and decrease their activity and life span (74). In oncology clinical practice, zoledronate is the most widely used bisphosphonate. This drug is administered intravenously, 4mg in 100ml 0,9% NaCl over 15 minutes, every three to four weeks. Zoledronic acid therapy should be continued, in absence of toxicity, at least for two years (65). In a trial designed to investigate the efficacy and safety of zoledronic acid for the treatment of bone metastases from breast cancer, 228 women with bone metastases were involved and zoledronic acid was compared to placebo, for one year. It was demonstrated that

zoledronic acid reduced the rate of SREs, the number of SREs per year, the risk of SREs and the time to develop a first SRE (75). A retrospective analysis from patients with bone metastases showed that in patients with highly aggressive or advanced disease, zoledronic acid had a positive effect on overall survival that was independent of SREs prevention (76). In addition to being anti-resorptive drugs, bisphosphonates can have both indirect and direct anti-tumor activity. Bisphosphonates can render the bone marrow a less favorable environment for cancer cell colonization by inhibiting the release of bone-derived growth factors during bone resorption, thus turning bone into a not so good "soil" to "seed" tumor cells (65). On the other hand, these drugs may interfere with the functions of bone marrow-derived cells (endothelial progenitor cells, mesenchymal cells, monocytes and macrophages), which, by enabling angiogenesis and the formation of pre-metastatic niches, have an important role in priming distant tissues for tumor metastases. Moreover, bisphosphonates may exhibit direct antitumor effects, especially when administered together with cytotoxic agents, having a synergistic effect. Finally, they can improve immune surveillance against neoplasia (77).

Therefore, antitumor activity of bisphosphonates may include inhibition of tumor cell adhesion and invasion, anti-angiogenic effects, immunomodulation, inhibition of tumor cell growth and induction of tumor cell apoptosis and reduction of the release of bone derived cytokines and growth factors.

1.5.2 Denosumab

The significant catabolic effects of RANKL on bone remodeling, in addition to its key pathogenic role played in many cancers, provides the rationale for the development of RANKL inhibitors, such as denosumab (AMG 162; Amgen), a subcutaneously injected neutralizing antibody against RANKL (78).

Denosumab prevents osteoclast activity and maturation from hematopoietic precursors. This non-cytotoxic IgG2 monoclonal antibody has an extremely high affinity for human RANKL and a long half-life, which allows less frequent dosing (79). It was developed to treat patients with skeletal pathologies mediated by osteoclasts, such as bone metastases, multiple myelomas, and cancer treatment induced bone loss (CTIBL) (56). Denosumab is a promising alternative for patients with bone metastases: recent studies

suggest that a monthly dose of denosumab (120mg/month) is superior to zoledronic acid (4mg/month) in delaying time to first, by over 8 months, and subsequent SREs in breast cancer patients (80). Therefore, RANKL inhibition with denosumab seems to be a better therapeutic option for prevention of SREs in patients with advanced cancer and bone metastases.

1.6 Bone markers and response to treatment

The processes of osteolysis and osteogenesis are associated with the release of distinct biochemical markers that are amenable to less-invasive measurement in blood or non-invasive measurement in urine (81). Serum levels and urinary concentrations of NTX, the amino [N]-terminal cross-linked telopeptide of type I collagen, and CTX, the carboxy [C]-terminal cross-linked telopeptide of type I collagen, reflect ongoing rates of osteolysis, whereas bone-specific alkaline phosphatase, bone ALP, and serum levels reflect ongoing rates of osteogenesis (81, 82). It is important to note that determinations of bone markers levels in urine should be normalized (e.g. by creatinine level) to control for the effects of hydration status and renal function (83).

Biochemical markers of bone metabolism reflect ongoing rates of bone resorption and formation as a whole (84). Therefore, their assessment does not provide information concerning individual lesion sites. Moreover, changes in bone marker levels are not disease specific, but are associated with alterations in skeletal metabolism independent of the underlying cause. Thus, the selection of the appropriate reference value is critical for data interpretation (81). Studies in patients with bone metastases from solid tumors suggest that alteration in the levels of bone resorption makers, such as NTX, can predict progression of bone metastases with greater specificity than ICTP and B-AP (85).

Based on the current evidence, NTX has the best-established correlation with clinical outcomes and response to bone-directed therapies, being a powerful predictor of skeletal complications (86). High baseline levels of NTX predict poorer outcomes in terms of duration of time to progression and survival in breast cancer patients with bone-only metastases and SREs occurs more frequently in breast cancer patients with raised bone resorption markers (87). The correlation between on-treatment NTX levels and risk of SREs or disease progression are especially striking (81). In 490 breast cancer patients

receiving zoledronic acid theraphy, NTX reduced from high levels (>100 nmol/mmol creatinine) to normal levels (<50nmol/mmol creatinine in younger patients; <64nmol/mmol creatinine in postmenopausal women (83)) within 3 months of zoledronic acid treatment in more that 75% of patients with high baseline NTX (86).

Return of NTX levels to normal during bisphosphonate treatment for bone metastases is associated in symptomatic response, a significant decrease in the rate of bone disease progression and a trend for a decreased incidence of fractures. In patients whose NTX levels normalized within 3 months, the subsequent risk of death was similar to that in patients with normal baseline NTX levels and was approximately 50% lower than in patients with persistently elevated NTX (88).

A correlation between NTX levels and clinical outcomes such as SREs, disease progression and death were analyzed and found to be statistically significant (85, 86).

Despite the results observed with bisphosphonates, approximately 30-50% of bisphosphonate-treated patients with bone metastases continue to experience skeletal complications (19). Therefore, there is a clear need to characterize the processes involved in the development and progression of bone metastases, in order to identify new therapeutic targets and improve outcome for these patients.

Furthermore, 25% of patients do not normalize NTX levels after bisphosphonate therapy. The mechanism of resistance underlying this phenomenon remains to be understood, but one possible explanation is the inter-individual variability due to any still unspecified genetic variation.

Protein tyrosine phosphorylation is the most important signal necessary to promote cell growth, proliferation, invasion and migration of normal and cancer cells, and thus it is not surprising that it is also involved in the process of bone metastases.

1.7 Protein tyrosine phosphorylation

Tyrosine phosphorylation is a key event on the communication between and within cells, cell shape and motility, decisions to proliferate versus to differentiate, cellular processes such as regulation of gene transcription, mRNA processing and transport of molecules in or out of cells (89). The control of protein tyrosine phosphorylation *in vivo* is regulated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases

(PTPs). Growing evidence indicates that the contribution of PTPs to control cell phosphorylation state is as relevant as that of PTKs (90).

1.7.1 Protein tyrosine phosphatases

The family of the currently identified almost 107 PTPs (91) comprises four classes, established based on the amino acid sequence of their catalytic domain (89), each with a range of substrate specificities: the classical receptor PTPs (RPTPs), the classical non-receptor PTPs (nrPTPs), the dual specificity PTPs (dsPTPs) and the low molecular weight PTPs (class II Cysteine-Based PTPs). The most significant trait of the protein tyrosine phosphatase superfamily is conservation of the CX_5R signature motif, which forms the phosphate binding loop in the active site (known as the P-loop or PTP-loop) (92).

Recent evidence suggests that members of the PTP family are key components of tumor growth and behaviour in various human cancers, exerting either putative oncogenic or tumor suppressive function, depending on the cellular context (93). At least 37 PTPs have been implicated in human cancer, with approximately equal proportions of oncogenic and tumor suppressor activities (94). Sastry and Elferink (95) reviewed the interplay of receptor tyrosine kinases and PTPs in cancer progression, focusing on PTP1B, PTPN11 and PTPN12, and defend that these three proteins can have different roles in cancer: the first has a dual role, and the other two opposite roles, tumor promotion and tumor suppression, respectively.

ACP1- LMW-PTP (Acid phosphatase 1 – Low molecular weight protein tyrosine phosphatase) has been described as a negative regulator of cellular proliferation induced by growth factors. However, recently, LMW-PTP was suggested as a positive regulator of tumor onset and progression in animal models (96), and has been shown to increase EphA2 (Ephrin A2 receptor)) receptor dephosphorylation, which is associated with many human cancers (97).

1.7.1.1 ACP1

The ACP1 gene is the only human member of the family of class II Cystein-Based PTPs (89), and encodes the low molecular weight protein tyrosine phosphatases (LMW-PTP; EC 3.1.3.2), a group of 18kDa proteins with no particular tissue specificity expression (90). The ACP1 gene, located on 2p25.3, is composed of seven exons interrupted by six introns spanning about 18kb of genomic DNA (98, 99). Human *ACP1* is genetically polymorphic, having three alleles, A, B and C that give rise to six genotypes – AA, AB, AC, BB, BC and CC. (99, 100). The 3 alleles (A, B and C) show almost 100% homology and differ by three single-nucleotide polymorphisms (SNPs) that affect both the total enzymatic activity and the ratio (101) between the two isoforms F (fast) and S (slow), that contribute to the enzymatic activity in a characteristic manner, thus explaining the different patterns seen in starch gel electrophoresis. In the A allele, aminoacid 105 is an arginine residue, while it is a glutamine in B and C. The other two SNPs do not change the encoded amino acid residues: the B allele differs from the A allele by a C-T transition on codon 41 (exon 4), and the C allele differs from both A and B alleles by a silent C-T transition on codon 43 (exon 3) that strongly affect the alternative mRNA splicing.

The gene has 13 transcripts encoding 5 proteins. Of the remaining 8 transcripts, 4 are thought to undergo nonsense mediated decay, a process that prevents the expression of truncated or erroneous proteins, 2 do not contain an open reading frame and 2 contain only intronic sequences (102). Two of the five translated proteins correspond to the main active isoforms of ACP1, ACP1_001 (NM_004300.3; electrophorectically *fast;* IF1) and ACP1_002 (NM_007099.3; electrophorectically *slow;* IF2). These two electrophorectically, kinetically and immunologically distinct isoforms, termed *fast* and *slow* based on their electrophoretic mobility (98), arise from mutually exclusive alternative splicing of exon 3 or 4 of the primary transcript Figure 1.3.

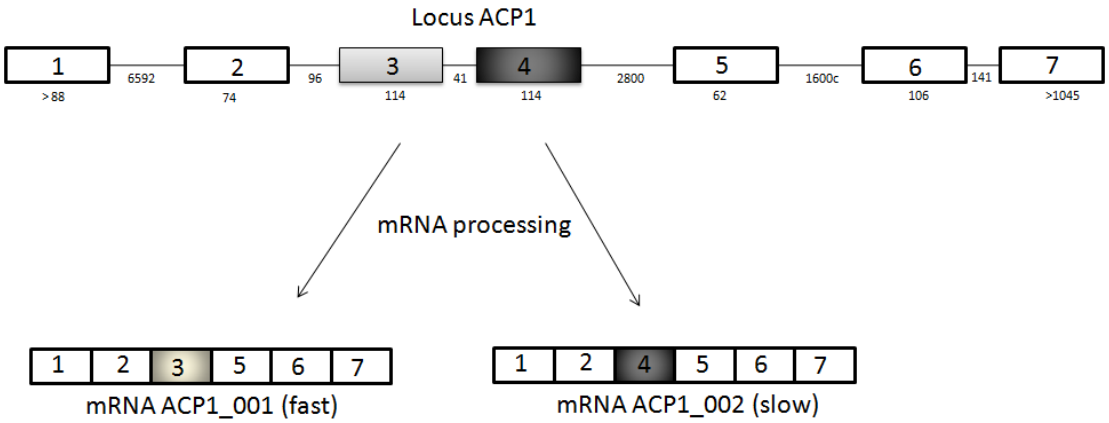

Figure 1.3 - ACP1 gene structure and mRNA splicing originating the two main isoforms: fast and slow. Adapted from Rudbeck et al (103).

These two exons, 3 and 4, encode the sequence for the aminoacid residues in positions 39-76 for both isoforms. The remaining exons, 1-2 and 5-7, are identical for the two isoforms, encoding aminoacid residues 1-38 and 77-157 (98, 103). Both human isoforms consist of a single peptide chain of 157 aminoacid residues. The aminoacid sequence is identical in the two isoforms except for the sequence 40-73, called the variable loop, with only 41% homology (100). This loop flanks the catalytic site and determine isoform specific in the binding to substrates and modulating ligands, suggesting the different roles for the two isoforms (104, 105). The active site, like in other phosphotyrosine-specific protein phosphatases, is deep enough to specifically catalyze the dephosphorylation of phosphotyrosine (pTyr) residues, preventing action on phosphoserine and phosphothreonine substrates (106).

The expression ratio of these isoforms seems to account for the phenotypic differences. The ratio of their activities differs markedly among genotypes, with *fast* B, the *fast* isoform produced by the B allele, being much more prominent than its *slow* counterpart (ratio 4/1), whereas the *slow* C isoform exhibits a much higher activity than the *fast* C (ratio 1/4). The two isoforms, *fast* A and *slow* A, occur in the ratio 2/1 (107).

These isoforms seem to be associated with different cell compartments. For many years, LMW-PTP was reported as an exclusively cytosolic enzyme, but Cirri et al. (104) demonstrated that LMW-PTP exists in two spatially and functionally separate pools: *fast* is associated with the cytoskeleton fraction whereas *slow* exists in the cytosolic pool.

The activity of LMW-PTP is regulated by tyrosine phosphorylation/dephosphorylation and reversible oxidation of cysteine residues. Specific aminoacid residues -Tyrosine 131 and Tyrosine 132; Cysteine 12 and Cysteine 17 - are critical in the regulation of LMW-PTP activity.

Tyr131 and Tyr132 are the two most important aminoacid residues for the regulation of LMW-PTP activity through phosphorylation. *In vivo*, the independent phosphorylation of these tyrosine residues can elicit different effects on the enzyme activity. Phosphorylation of Tyr131, the main phosphorylation site, is essential for several functions of LMW-PTP, mainly control of cell growth and adhesion, whereas intriguingly on the contrary, the phosphorylation in position 132 could result in negative regulation of the enzyme. Furthermore, the alternative phosphorylation of Tyr131 and Tyr132 can provide a fine tuned regulation of LMW-PTP activity in different physiological conditions.

The other crucial aminoacid residues involved in key regulatory mechanisms are Cys12 and Cys17. The thiol groups of neighboring Cys12 and Cys17 are oxidized by both H_2O_2 and NO to form a disulfide bond, which protects the initially formed cysteine sulfenic acid (Cys-SOH) from further irreversible oxidation to sulfinic and sulfonic acids (106).

The consequent enzyme inactivation is reversible and recovery of the LMW-PTP function was observed in the presence of reducing agents, such as dithiothreitol or reduced glutathione, or after removal of oxidative conditions. *In vivo*, endogenous H_2O_2 is generated in numerous conditions of oxidative stress, such as growth factor signal transduction. Therefore, the cellular redox balance can strongly influence PTPase activity (108, 109).

1.7.2 LMW-PTP interaction with cancer-associated molecules

Protein phosphorylation plays key roles in many physiological processes and is often deregulated in pathological conditions (110) such as cancer, which may be considered a pathology of deregulated signal transduction. The relevant molecules that are apparently regulated by LMW-PTP in cancer progression are Platelet Derived Growth Factor-Receptor (PDGF-R) (111), p190RhoGAP (97, 112), Ephrin A2 receptor (EphA2) (113), Src (114, 115), β-catenin (116). LMW-PTP interaction with the different molecules is resumed in Figure 1.4.

1.7.2.1 p190RhoGAP

Cell-cell adhesion, a paramount feature in oncogenesis, is regulated by multiple mechanisms, and the downstream effects of the Rho family GTPases has been recognized as a key player in this process. Rho family GTPases alternate between active (GTP-bound) and inactive (GDP bound) conformations. Rho has a major role in directing actin dynamics (97) promoting, specifically, stress fibers generation (117). Rho upstream regulators include GTPase-activating proteins (GAPs), guanosine nucleotide exchange factors (GEFs) and RhoGTPases dissociation inhibitors (RhoGDIs) (118). One of the important GAPs is p190RhoGAP, which is involved in cytoskeleton rearrangement and seems to be one of LMW-PTP cytoskeletal associated fraction specific substrates (90, 112, 119). It has been shown by Vincent et al. (120) that inactivation of RhoGAP is an effective means of promoting Rho-mediated cellular processes. Through dephosphorylation of p190RhoGAP, LMW-PTP may be involved in the regulation of the small GTPase Rho, potentiating its action, and the consequent cytoskeleton rearrangement.

This relationship between p190RhoGAP and LMW-PTP was also shown in K-Ras transformed cells (Kirstein-Ras-transformed Normal Rat Kidney fibroblast) (121). Nox-1 generates ROS that oxidize and inactivate LMW-PTP, resulting in the accumulation of tyrosine-phosphorylated active p190RhoGAP. This p190RhoGAP causes down-regulation of Rho, possibly leading to the deregulation and hindrance of actin stress fibers formation and focal adhesion assembly (121). These cellular alterations lead to changes in the adhesion and migratory potential of the cells.

Given migration is one of the hallmarks of tumor spreading, invasion and metastization, and elevated expression levels of RhoA have been correlated with tumor stage or enhanced metastasis in several tumors, including breast cancer (122), the action of RhoA can be potentiated through LMW-PTP. Furthermore, a relationship between EphA2, a molecule that has been largely associated with tumorigenesis, p190RhoGAP and cellular adhesion has been established through Src and LMW-PTP

1.7.2.2 EphA2

The oncogenic activity of LMW-PTP is closely related to changes in the expression and function of its substrate EphA2 observed in tumor cells both *in vitro* and *in vivo*. The EphA2 receptor tyrosine kinase is overexpressed and dephosphorylated in many human carcinomas and melanomas, especially in aggressive and metastatic types of cancer. EphA2 tyrosine phosphorylation is a crucial feature that strongly influences the oncogenic potential of this kinase (113). Unphosphorylated EphA2 exerts strong oncogenic actions. The tyrosine phosphorylated EphA2 , found mainly in normal cells, activates downstream events that lead to inhibition of cell growth and migration and invasion. (123). In tumor cells the overexpressed LMW-PTP acts as a critical regulator of EphA2 tyrosine phosphorylation. Therefore, LWM-PTP triggers cellular neoplastic transformation and promotes cancer progression through high levels of unphohsphorylated EphA2 tyrosine kinase, which functions as an essential downstream component of this oncogenic signaling pathway (106). In fact, LMW-PTP overexpression alone is sufficient to promote cellular transformation and accelerates both tumor growth and required implantation time *in vivo* (93).

The recruitment of LMW-PTP by EphA2 prevents the phosphorylation of p190RhoGAP, upregulating RhoGTP levels, and ultimately leading to destabilization of cell-cell adhesion, weaker cell-matrix adhesion and to more invasive cells (97). These results suggest that the upregulation of LMW-PTP leads to the impairment of adhesion and to a more aggressive migratory phenotype, considering that high levels of LMW-PTP can be taken as a marker for tumor aggressiveness and unfavourable outcome (93). In fact, EphA2 is the main substrate of LMW-PTP in tumors, confirming that the oncogenic potential of this phosphatase is linked to EphA2 dephosphorylation (96).

1.7.2.3 Src

Src contributes to the maintenance of normal cell homeostasis and to a vast number of physiological functions including cell proliferation and survival, regulation of cytoskeleton, cell shape control, maintenance of normal intracellular contacts, matrix adhesion dynamics, motility and migration (115). Src is a inducer of epithelial-mesenchymal transition because an elevated level of Src induces cell-cell junction

destruction through both phosphorylation and degradation of E-cadherin and up-regulation of mesenchymal markers such as vimentin and N-cadherin (115).

Src activity is regulated by oxidation and by phosphorylation of two main Tyr residues: A-loop Tyr416 and C-term Tyr527. Src is inactive when is in its reduced state and Tyr527 is phosphorylated. It happens mainly in resting adherens cells, under normoxia that are non-invading cells. Under stimulation with growth factor and cytokines in "early cell adhesion" Src became moderately active through dephosphorylation of Tyr527, phosphorylation of Tyr416 and maintaining its reduced state. In invading cells under hypoxia, Src is fully activated by dephosphorylation of Tyr527, phosphorylation of Tyr416 and by oxidation through the formation of disulphide bound (115).

LMW-PTP is associated with Src activation by means of Tyr527 dephosphorylation and is also implicated in its inactivation, by acting on the p-Tyr416 of the A-loop.

During osteoblast differentiation, LMW-PTP produces a strong activation of Src medited by fast dephosphorylation of the Tyr527, followed by a slower deactivation of this kinase via dephosphorylation of the Tyr416 (114).

Fully activated Src strictly associates with LMW-PTP, inducing the tyrosine phosphorylation through strongly phosphorylation of both Tyr131 and Tyr132 (106, 124) activating the phosphatase, which in turn promotes Src inactivation (114). The positive feed-back loop proposes LMW-PTP as a prime candidate in the dynamics of Src activation after cellular stimuli (115).

The regulation LMW-PTP and Src and their consequent interaction are an example of a feed-forward loop, that are a constant finding in redox signaling: LMW-PTP is active on the accessible Tyr416 of Src, thereby inactivating the kinase, and this event is probably correlated with Tyr 416 hyperphosphorylation and Src activation, leading to a further feed-forward redox-based loop (114, 115).

Src and protein tyrosine phosphatases are instrumental in bone metabolism (125): results from Zambuzzi et al showed that Src activity, but not expression, was significantly altered during osteoblast differentiation and these changes are, apparently, modulated by the action of LMW-PTP in this kinase (114). Also, de Souza Malaspina et al showed that osteoblastic cells express LMW-PTP in a time-dependent manner, and its activity during osteoblast differentiation was rigorously modulated, progressively increasing (126).

Therefore, regulation of Src activity by LMW-PTP can be an important link between LMW-PTP and bone metabolism since Src is crucial for the regulation of osteoclastic activity, which, as previously described, is dependent on osteoblast release factors such as RANKL.

Taken together, these facts suggest that LMW-PTP plays a critical role in the osteoblast machinery, being an important molecule in osteoblast biology, bone formation and possibly in osteoclasts metabolism (126).

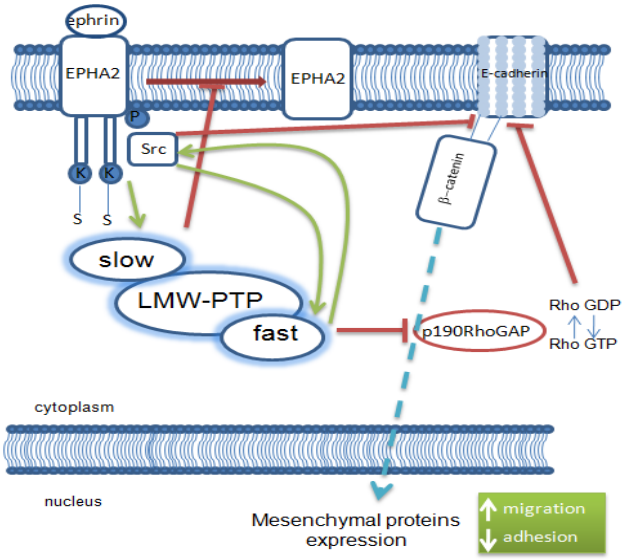

Figure 1.4 - Interactions between LMW-PTP and cancer associated molecules. LMW-PTP inhibits important molecules that may be involved in cancer processes. Dephosphorylation of EPHA2 by LMW-PTP *slow* isoform increases tumor growth and metastatic potential, promoting transformation- *Fast* isoform dephosphorylates and inhibits p190RhoGAP and consequently potentiates Rho action which, through E-cadherin, destabilizes adherens junctions. An important molecule in this process is Src: Src can be activated by ROS and activated Src associates with the *fast* isoform of LMW-PTP. This association induces tyrosine phosphorylation and activation of LMW-PTP, which in turn promotes Src activation. Src is able to inhibit the association between E-cadherin and β-catenin, promoting β-catenin signaling action and consequently increasing the expression of mesenchymal proteins, enhancing migration and decreasing cell adhesion.

1.7.3 ACP1 and cancer

ACP1 has been associated with different pathologies, eg developmental disturbances and haemolytic favism (107), Systemic Lupus Erythematosus (127), obesity-related hypertension (128), hypertension (129) and cancer (130, 131).

We have previously reported a positive association between the *fast* isoform of *ACP1* and human cancers, mainly cervix and breast (130). On the other hand, Spina et al. (131) have shown that the protective effect of the *slow* isoform in colon cancer patients prevails over the predisposition effect of the *fast* isoform. The apparent disparity between these studies may be related to tumor type or stage.

It is presently unknown if the differential expression of the two isoforms due to the ACP1 polymorphism may have different roles in tumor growth and cancer progression, or if different types of cancer cells express different amounts of the LMW-PTP isoforms. There is a clear need to evaluate different stages of tumor progression and metastization in order to understand the relationship between these polymorphisms and the pathophysiology of the disease, and *in vitro* studies have been conducted in order to better understand the underlying mechanisms. Chiarugi et al. (96) have shown that, while LMW-PTP negatively regulates growth factor-mediated proliferation in NIH3T3 mouse embryonic fibroblast cultures, in animal models it acts as a positive regulator of tumor onset and growth (96). These authors also show that despite that fact that LMW-PTP overexpressing cells are endowed with enhanced *in vitro* adhesion and mobility, their engrafts did not lead to metastasis. Malentacchi et al. (132) evaluated the expression levels of LMW-PTP mRNA in different human carcionomas – breast, colon, lung and a group of neuroblastoma samples as an example of neuroendocrine cancer. Results strongly suggest a common pattern, by which an increase of LMW-PTP expression, irrespective of the isoform, is observed in most tumour samples – breast and colon, but not lung cancers. LMW-PTP protein content was in agreement with the observed increase in mRNA, confirming that the overexpression of LMW-PTP mRNA leads to LMW-PTP protein overproduction (132). Based on these results, the authors concluded that the *ACP1* gene can be considered an oncogene.

This overexpression of LMW-PTP in different types of cancer is consistently found in tumor cells that have high levels of unphosphorylated EphA2. LMW-PTP might regulate EphA2 expression, function or both and, indeed, ectopic expression of LMW-

PTP causes dephosphorylation and upregulation of EphA2 (113, 123). Kikawa et al. also confirmed that the “oncogenic activities” of LMW-PTP require EphA2, since antisense or antibody-based inhibition of EphA2 expression prevents LMW-PTP-mediated malignant transformation (113), and this overexpression is sufficient to confer transformation upon non-transformed epithelial cells.

Thus, there is multiple evidence that LMW-PTP is also involved in carcinogenesis, although its influence cannot be unequivocally classified as pro- or anti-oncogenic.

In view of possible targeted intervention targeted at LMW-PTP, it is particularly important to consider the expression of the main LMW-PTP isoforms (*fast* and *slow*) in tumor tissues. Although the pathophysiology roles of the human LMW-PTP isoforms still need to be further elucidated in greater detail, the identification of LMW-PTP inhibitors can be considered as a new approach for developing novel antitumor strategies (106).

The search for drugs targeted at LMW-PTP has only recently begun; efforts have been made to target the enzyme in the search of new targets for hyperproliferative cell disorders disorders. Aplidin is a depsipeptide with a potent antineoplastic effect, has been recognized to be mediated, at least, in part LMW-PTP inactivation. Aplidin does not exert a direct inhibitory effect on the enzyme the LMW-PTP inactivation appears to be caused by the cellular oxidative stress induced following aplidin treatment (133).

1.8 Objectives

Due to the controversial role of LMW-PTP in tumor behavior and progression and the paucity of information regarding its isoforms, the aims of this work were:

1. to study the molecular mechanisms and relevance of the expression of the two main LMW-PTP isoforms in tumor growth and progression, namely bone metastization.

2. to evaluate the response to bisphosphonates therapy in patients with bone metastasis, according to ACP1 genotypes.

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Chapter 2

Characterization of Low Molecular Weight Protein Tyrosine Phosphatase Isoforms in Human Breast Cancer Epithelial Cell Lines

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2 Characterization of Low Molecular Weight Protein Tyrosine Phosphatase Isoforms in Human Breast Cancer Epithelial Cell Lines

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Characterization of Low Molecular Weight Protein Tyrosine Phosphatase Isoforms in Human Breast Cancer Epithelial Cell Lines

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Abstract. *Background: Low molecular weight protein tyrosine phosphatase (LMW-PTP) is a polymorphic protein with two major isoforms whose role in tumorigenesis is currently controversial. Materials and Methods: We characterized LMW-PTP genotype, isoform expression and activity in six different human breast cancer epithelial cell lines (ZR75, MDA-MB-231, MDA-MB-231BO, MCF7, MDA-MB-231BO2, MDA-MB-435) and compared them with MCF10A, a normal breast epithelial cell line. Results: mRNA expression of the slow isoform was increased in almost all breast cancer cell lines and that of the fast isoform was reduced ($p<0.05$) in all breast cancer cell lines. Regarding enzymatic activity, only MCF7 had significantly lower ($p<0.05$) LMW-PTP activity compared to MCF10A. Conclusion: Since these are novel and previously unreported findings, we propose that the differential expression of LMW-PTP fast and slow isoforms suggests their opposite roles in the tumorigenic process, with the fast isoform being anti-oncogenic and the slow isoform being oncogenic.*

Protein tyrosine phosphorylation is a key mechanism through which cells control vital functions such as cell growth, proliferation, motility and gene expression (2). This process is controlled by two groups of enzymes, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). PTPs are a family of 107 enzymes that comprise of four classes (2). All enzymes share a common CX5R active site motif and an identical catalytic mechanism that is based on the participation of a crucial cysteine residue (25).

Low molecular weight protein tyrosine phosphatases (LMW-PTP, EC 3.1.3.2) are a group of 18-kDa enzymes, with no particular tissue specificity (20), that belong to the class II cysteine-based PTPs, and are represented in the human genome by a single gene, *ACP1*, located on 2p25.3 (21). *ACP1* is polymorphic and has three different alleles, A, B, and C, with almost 100% homology, differing only by three single nucleotide polymorphisms (SNPs). These polymorphisms affect both total enzymatic activity (13) and the ratio (12) between the two most abundant LMW-PTP isoforms, named *fast* (accession number NM_004300.3; ACP1_001) and *slow* (accession number NM_007099.3; ACP1_002), according to their electrophoretic mobility (7). These isoforms arise from mutually exclusive alternative splicing of either exon 3 or 4, and their protein sequence differs by a 42-amino acid internal sequence (19). There is evidence that this enzyme interacts with some molecules involved in tumorigenesis, *e.g.* platelet derived growth factor receptor (PDGFR) (8), p190RhoGAP (15), Ephrin-A2 receptor (EPHA2) (16) and β-catenin (24).

Overexpression of total LMW-PTP has been observed in many oncogene-transformed epithelial cell lines and is sufficient to transform non-transformed epithelial cells (16). The *in vivo* role of LMW-PTP in tumorigenesis has been analyzed by Chiarugi *et al.* (10), who evaluated the effect of overexpression of both total LMW-PTP and a dominant negative LMW-PTP on sarcoma development in nude mice. Total LMW-PTP was shown to be a positive regulator of both tumor onset and development *in vivo*. However, Malentacchi *et al.* analyzed a panel of human breast, colon and lung cancer surgical samples and their paired adjacent non-affected tissues, and observed that breast and colon cancer, but not lung cancer, exhibited increased mRNA levels for *LMW-PTP* (18).

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Key Words: Protein tyrosine phosphorylation, LMW-PTP isoforms, genotype, mRNA expression, enzymatic activity.

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Given these discrepancies, and the fact that these studies only investigated total LMW-PTP, it is important to investigate the role of the two most abundant isoforms of LMW-PTP in different human cancer cell lines, and characterize their differential expression in these cells.

Materials and Methods

Cell culture. We characterized six different human breast cancer epithelial cell lines with respect to LMW-PTP genotype, isoform expression and activity. MCF10A, a spontaneously immortalized but non-transformed human breast epithelial cell line isolated from fibrocystic disease, is considered to be a normal breast epithelial cell line and was used as a comparator. All cell lines except MDA-MB-231 BO, MDA-MB-231 BO2 and ZR-75, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231 BO, MDA-MB-231 BO2 and ZR-75 were kind gifts from the Division of Endocrinology and Metabolism (University of Texas Health Science Centre, San Antonio, Texas, USA), INSERM Research Unit 403 (Faculté de Medecine Laennec, Lyon, France), and the University of Virginia, USA, respectively. MCF10A was cultured in Clonetics mammary epithelial cell growth medium (MEGM) supplemented with BulletKit (CC-3150) (Lonza, Basel, Switzerland) and 100 ng/ml cholera toxin. MDA-MB-231 is derived from a breast adenocarcinoma and was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. MDA-MB-231 BO and MDA-MB-231 BO2 are clones from the original cell line MDA-MB-231 isolated from bone metastasis. The BO clone was cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The BO2 clone was cultured in RPMI-1640 supplemented with 0.25 mg/ml G418, 10% FBS and 1% penicillin/streptomycin. MCF7 is derived from an adenocarcinoma and was cultured in Eagle's minimum essential medium supplemented with 1.0 mM sodium pyruvate , 0.1 mM non-essential amino acids , 1.5 g/l $NaHCO_3$, 0.01 mg/l bovine insulin, 10% FBS and 1% penicillin/streptomycin. MDA-MB-435 and ZR-75 are derived from ductal carcinomas and were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

Genotyping. Cells were cultured in 6-well plates and incubated in serum-free medium for 48 h prior to experiments. DNA was isolated from cells at 90% confluence using the Easy Spin® Nucleic Acid Extraction kit (Citomed, Lisbon, Portugal) and quantified with Nanodrop (ThermoScientific, Waltman, MA, USA). *ACP1* genotypes were determined by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP). Two hundred nanograms of genomic DNA were used with the following primers: forward 5' CGA TCA CCC ATT GCA GAA G 3' and reverse 5' CCA TGA TTT CTT AGG CAG CTC 3'. PCR conditions were 94˚C 30 s; 51˚C 30s; 72˚C 45s, 35 cycles, and a final extension at 72˚C for 5 min. The amplified fragment, of 400 bp, was digested with two different restriction enzymes, *Hin6I* and *MspA1*.

LMW-PTP mRNA expression. Cells were cultured in 6-well plates and incubated in serum-free medium for 48 h prior to experiments. Total cellular RNA was isolated from cells at 90% confluence with the RNeasy mini kit (Qiagen, Foster City, CA, USA). RNA was quantified by absorbance at 260 nm, and purity determined by absorbance at 280 and 310 nm with NanoDrop (ThermoScientific). RNA (1 μg) was converted into cDNA using the QuantiTect® Reverse Transcriptase kit (Qiagen, Foster City, CA, USA). An aliquot (0.2 μl) of the cDNA was amplified in an ABIPrism 7000 real-time RT-PCR unit using the following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA): ACP1 total (Hs00962877_m1); *ACP1 fast* isoform (Hs00964348_g1); *ACP1 slow* isoform (Hs00246642_m1). Results were normalized to real-time RT-PCR of Glyceraldeyde 3-phosphate dehydrogenase (GAPDH) using the Human GAPDH Endogenous Control (Applied Biosystems).

Table I. *Acid phosphatase 1 (ACP1) genotype of human breast epithelial cell lines*.

Cell line	Genotype
MCF10A	BB
MDA-MB-231	AB
MDA-MB-231 BO2	AA
MDA-MB-231 BO	AB
MDA-MB-435	AA
MCF7	AB
ZR-75	BB

Enzymatic activity. Cells were cultured in 96-well plates and incubated in serum-free medium for 48 h prior to experiments. *Ex vivo* activity of LMW-PTP was measured in cells at 60%-70% confluence according to the method of Balcerczyk *et al*. (4). Briefly, lysis buffer, containing 10 mM *p*-nitrophenyl phosphate and 0.1% Triton X-100 in 0.1 M sodium acetate, 10 mM EDTA (pH 5.5) was added to the cells for 2 h. Samples were alkalinized by adding 1 M NaOH and the absorbance of *p*-nitrophenol was then measured at 405 nm. Results were normalized to total protein content, determined by Precision Red™ Advanced Protein Assay Reagent (Cytoskeleton, Denver, CO, USA). This method measures total LMW-PTP enzymatic activity. There is currently no method capable of differentiating between the activity of the different isoforms.

Statistical analysis. All data are expressed as the mean±standard deviation. Significance was established by the Student's *t*-test or ANOVA and *post-hoc* Sidak test, where appropriate. Differences were considered significant at $p<0.05$.

Results

ACP1 genotype of human breast epithelial cell lines. *ACP1* has three different alleles, A, B, and C, thus giving rise to six possible genotypes. *ACP1* genotype of each of the studied cell lines is presented in Table I, showing that only three out of the six possible *ACP1* genotypes are present, AA, AB and BB.

LMW-PTP mRNA expression in human breast epithelial cell lines. Figure 1 shows the relative expression of total LMW-PTP and its *fast* and *slow* isoforms in the studied tumor cell

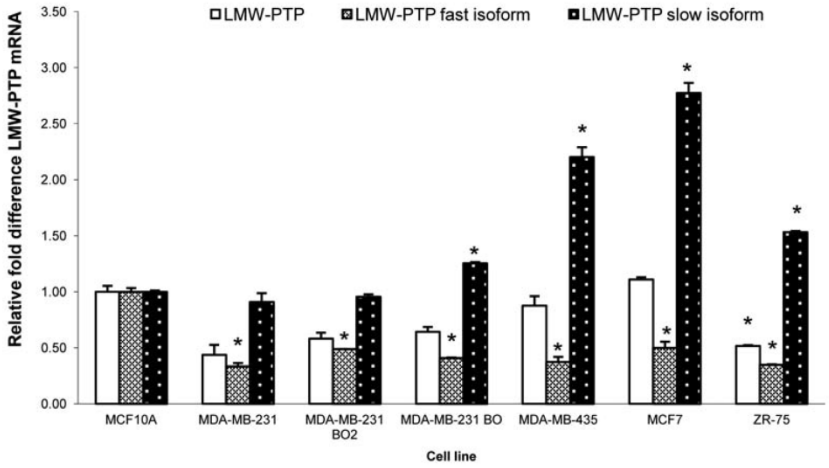

Figure 1. *mRNA expression of low molecular weight protein tyrosine phosphatase (LMW-PTP) and its isoforms in human breast cancer epithelial cell lines. Total RNA was isolated, cDNA was prepared and real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described in the Materials and Methods. Results were normalized to real-time RT-PCR of glyceraldeyde 3-phosphate dehydrogenase (GAPDH) and relative fold differences were calculated in comparison to MCF10A (non-tumor cell line), which was taken as 1.00. Results are expressed as the mean±SD of triplicates and are representative of three independent experiments. *p<0.05 compared to MCF10A.*

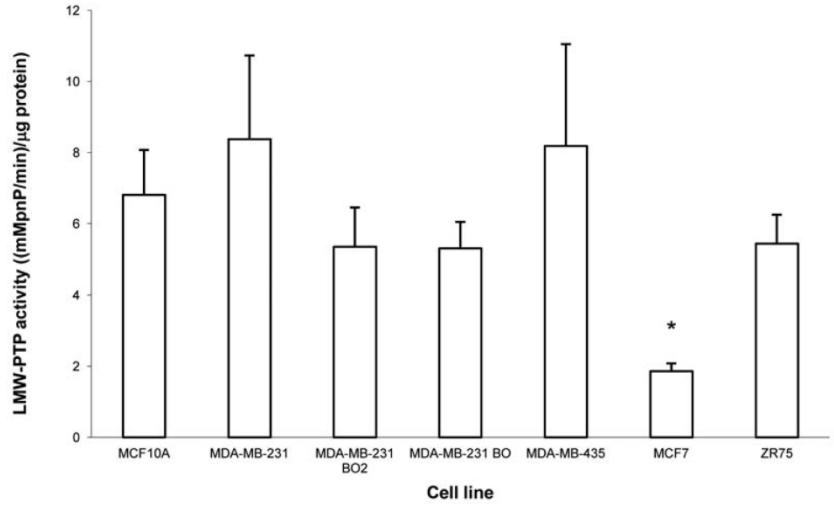

Figure 2. *Low molecular weight protein tyrosine phosphatase (LMW-PTP) activity in human breast epithelial cell lines. *p<0.05 compared to MCF10A.*

lines compared to MCF10A. There was an increased expression of the *slow* isoform in almost all breast cancer cell lines and a reduced expression of the *fast* isoform in all breast cancer cell lines. Expression of total LMW-PTP was only reduced in the ZR-75 cell line.

LMW-PTP activity in human breast epithelial cell lines. Enzymatic activity of LMW-PTP depends not only on the *ACP1* genotype but also on the presence of inhibitors and activators (17) . The results show that only MCF7 had a lower LMW-PTP activity when compared to MCF10A (Figure 2).

Discussion

There are various studies associating genetic polymorphisms of *ACP1* with different pathologies, *e.g.* developmental disturbances and hemolytic favism (6), systemic lupus erythematosus (14), obesity-related hypertension (3), hypertension (11) and cancer (1, 23). *ACP1* genotyping in patients with different types of cancer has shown a positive association of cancer with genotypes carrying the B allele (1) and a decrease of genotypes carrying the C allele (23). In our cell lines, only three out of the six possible *ACP1* genotypes were present, AA, AB and BB. These are the most frequent genotypes and the ones that seem to be positively-correlated with cancer (1, 23).

Malentacchi *et al.* (18) studied the expression profile of LMW-PTP in a large panel of human tumors and their paired adjacent normal tissue and concluded that overexpression of *LMW-PTP* mRNA leads to over-production of LMW-PTP protein. Their results suggest that LMW-PTP overexpression occurs in breast cancer and is correlated with the aggressiveness of the tumor. The fact that the expression of total LMW-PTP was only lower in the ZR-75 cell line when compared to MCF10A, with no changes in the other tumor cell lines, may reflect the presence of all other isoforms, since *fast* and *slow* are not the only isoforms of LMW-PTP (19, 21). Therefore, the fact that total LMW-PTP expression is not different from MCF10A, although there is an alteration in both the *fast* and *slow* isoforms expression, can be due to the contribution of the others isoforms.

However, there was an increase of the *slow* isoform expression in almost all cancer cell lines and a reduction of expression of the *fast* isoform in all tumor cell lines, compared to MCF10A. This leads us to suggest that this differential expression may be associated with the tumorigenic potential of the cells. A possible mechanism that could explain the pro-oncogenic potential of the *slow* isoform is the de-phosphorylation potential of membrane receptor proteins this isoform has (8). Given previous reports suggesting that de-phosphorylated EPHA2 favors transformation of normal epithelial cells and influences tumor growth (22), it may be hypothesized that the *slow* isoform can increase the tumorigenic potential through de-phosphorylation of EPHA2. Regarding the decrease in mRNA expression of the *fast* isoform, this can reduce the phosphorylation status of Ras homolog gene family, member A (RhoA), through p190RhoGAP, rendering it more inactive (GDP-bound conformation). This modification alters cell adhesion and, consequently, the migratory ability of the cells. Therefore, LMW-PTP isoforms may be involved in the tumorigenic process by interfering with cell growth, adhesion and migration, which are hallmarks of cancer. Taken together, these results suggest a potential role of LMW-PTP *fast* and *slow* isoforms as prognostic markers and possible therapeutic targets in cancer.

Regarding LMW-PTP enzymatic activity, our results showed that only the MCF7 cell line had a lower LMW-PTP activity when compared to MCF10A. This is not consistent with the mRNA expression, which showed that only the ZR-75 cell line has a lower total LMW-PTP expression. Therefore, it would be expected that this cell line would have a lower total enzymatic activity. We have no straightforward explanation for these results since they have not been reported previously. We can hypothesize that in the ZR-75 cell line, the LMW-PTP protein can be directly activated or stabilized, its mRNA stabilized or the mRNA translation rate increased. Moreover, and regarding post-translational modifications, phosphorylation of Tyr131 and Tyr132 can induce an increase of LMW-PTP activity (9). Given LMW-PTP is finely-regulated in tumor cells, several of these mechanisms, either alone or synergistically, may be sufficient to explain the observed differences.

Regarding MCF7 results, MCF7 is a non-metastatic breast tumor cell line that seems to have different characteristics compared to the other studied cell lines. These cells grow more slowly, more clustered and have epithelial characteristics, since they do not express vimentin nor N-cadherin, two important mesenchymal markers (5). These intrinsic characteristics of the cells can interfere with LMW-PTP activity measurement since substrate availability may be low due to limited access to all cells: although the number of cells is the same, the fact that they grow closely in clusters may hinder the efficacy of cellular lysis and consequently limit substrate access. The fact that this cell line exhibits a more epithelial-like phenotype, similar to MCF10A, unlike ZR-75, means that other factors, such as purines, folic acid and pirimidines (17), may be involved in the regulation of LMW-PTP activity. Moreover, our results suggest novel functions of LMW-PTP isoforms in tumorigenesis, showing that the expression of these isoforms is different among human breast cancer epithelial cells, with an overexpression of the *slow* isoform in almost all of the studied tumor cell lines and reduced expression of the *fast* isoform in all tumor cell lines. This leads us to propose that these two isoforms have opposing roles in the tumorigenic process, with the *slow* isoform being oncogenic and the *fast* isoform anti-oncogenic, which can explain the previous contradictory findings regarding the role of LMW-PTP in cancer. Furthermore, we propose that LMW-PTP isoforms may be considered prognostic markers of the tumorigenic process, and possible therapeutic targets. Given these are novel and previously unreported findings, we are currently conducting studies to explore the possible differential role of LMW-PTP isoforms in the tumorigenic process.

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Chapter 3

Low molecular weight protein tyrosine phosphatase isoforms regulate breast cancer cell migration through a RhoA dependent mechanism

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3 Low molecular weight protein tyrosine phosphatase isoforms regulate breast cancer cell migration through a RhoA dependent mechanism

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3.1 Abstract

Low molecular weight protein tyrosine phosphatase (LMW-PTP) has been associated with cell proliferation control through dephosphorylation and inactivation of growth factor receptors such as PDGF-R and EphA2, and with cellular adhesion and migration through p190RhoGap and RhoA. We aim to clarify the role of two main LMW-PTP isoforms in breast cancer tumorigenesis. We used a siRNA-mediated loss-of-function in MDA-MB-435 breast cancer cell line to study the role of the two main LMW-PTP isoforms, *fast* and *slow*, in breast cancer tumorigenesis and migration. Our results show that the siRNAs directed against total LMW-PTP and LMW-PTP *slow* isoform enhanced cell motility in an invasive breast cancer cell line, MDA-MB-435, with no changes in the proliferation and invasive potential of cells. The total LMW-PTP knockdown caused a more pronounced increase of cell migration. Supression of total LMW-PTP decreased RhoA activation and suppression of the LMW-PTP *slow* isoform caused a small but significant increase in RhoA activation. We propose that the increase or decrease in RhoA activation induces changes in stress fibers formation and consequently alter the adhesive and migratory potential of cells. These findings suggest that the two main isoforms of LMW-PTP may act differentially, with the *fast* isoform having a more prominent role in tumor cell migration. In addition, our results highlight functional

specificity among LMW-PTP isoforms, suggesting hitherto unknown roles for these proteins in breast cancer biology. Novel therapeutic approaches targeting LMW-PTP, considering the expression of these two isoforms and not LMW-PTP as a whole, should be investigated.

3.2 Introduction

Protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs) regulate the reversible phosphorylation of tyrosine residues in proteins, thus controlling vital physiological processes [1].

In humans, class II cystein-based PTPs are represented by members of the Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP) family, which are widely expressed, with no particular tissue specificity. LMW-PTP is encoded by the ACP1 (acid phosphatase locus 1) gene, located at 2p25, spanning 7 exons and 6 introns. The enzyme has two main isoforms, IF1 (fast) and IF2 (slow), both small enzymes consisting of only 157 aminoacid residues and with a molecular weight of 18kDa. The aminoacid sequence shows that the two isoforms arise from alternative and mutually exclusive splicing of exon 3 or 4 [2].

The 2 isoforms may have different roles in the progression of oncologic pathology [2,3]: the fast isoform is involved on migration, invasion and cell adhesion, catalysing the transformation of different substrates after platelet derived growth factor receptor (PDGF-R) stimulation, whilst the slow isoform, acting directly on PDGF-R, has growth factor receptors as substrates, eg platelet derived growth factor receptor (PDGF), leading to a decrease of cellular growth via its dephosphorylation [3]. LMW-PTP has been largely considered a negative regulator of growth factor-induced cell proliferation, although in some instances it acts as a positive regulator. Some proteins, such as Ephrin Receptor A2 (EphA2), seem to be involved in the regulation of carcinogenesis by LMW-PTP. Eph receptors are a family of receptor tyrosine kinases that have been shown to be overexpressed in a large number of cancers [4,5]. It is known that LMW-PTP has the potential to dephosphorylate EphA2 rendering it negatively regulated, which can increase transformation of normal epithelial cells, regulate tumor cell growth, survival, migration and invasion [1]. p190RhoGAP, a protein involved in the regulation of cytoskeleton rearrangement, is also dephosphorylated by LMW-PTP, with the

consequent effect on RhoA [3]. This observation correlates with the influence of LMW-PTP expression on phenomena such as cell adhesion, spreading and migration [3].

Fang et al defend that enhanced RhoA activity is apparently regulated by enhanced LMW-PTP phosphatase activity and inhibition of tyrosine phosphorylation of p190RhoGAP, ultimately leading to the destabilization of cell-cell adhesion. They describe that overexpression of EphA2 promotes destabilization of adherens junctions through the axis LMW-PTP – p190RhoGAP – RhoA [6].

Given the controversial role of LMW-PTP in tumor growth and progression, this study aimed at clarifying the importance of LMW-PTP isoforms in breast tumor cell growth, migration and invasion.

Our results show that blocking total LMW-PTP and its slow isoform by siRNA in the MDA-MB-435 cell line, a breast cancer invasive cell line, results in increased migratory potential, which our results suggest to occur through RhoA. Therefore, we suggest that the control of LMW-PTP expression, with the consequent balance of RhoA activation, may be a pathway through which the migratory potential of cells is regulated, indicating that LMW-PTP may have an important role in cell migration. There seems to be a differential effect of the two isoforms, with the fast isoform having a more important role in cell migration, which may indicate that this isoform is involved in a later stage of tumor development and the slow isoform in an earlier stage.

3.3 Methods

3.3.1 Cell culture

The breast cancer cell line MDA-MB-435 was obtained from the American Type Culture Collection (ATCC number HTB-129) and grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco – Foster City, CA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (10 μg/ml), in a humidified atmosphere of 5% CO2 at 37ºC. All experiments were performed on cells with population doublings between 84 and 100. Knockdown efficiency was confirmed in cells incubated in serum-free DMEM for 48h prior to RNA extraction and assessment of LMW-PTP activity. However, and since for proliferation, migration and invasion assays

cells have to be maintained in complete growth medium according to manufacturer's instructions, all experiments were performed in complete growth medium, in order for the results to be comparable.

3.3.2 Knockdowns of LMW-PTP isoforms

Five different siRNA sequences were designed to specifically knockdown the total protein (KD LMW-PTP) (GeneBank NC_000002.11) (CI#1 5'-GCA AGA CAG ATT ACC AAA GAA-3'; CII#2 5´-GCC TGT TGS GAC TTA GAT AAT-3´; CII#3 5´CTA TGT ATG GAT GAA AGC AAT3´; CV#5 5´-GAA CTA CTT GGG AGC TAT GAT-3´) and the *slow* isoform (KD LMW-PTP slow) (GeneBank accession number NM_007099.3) (CIV#4 5´- GCC CAT AAA GCA AGA CAG ATT-3'). One scramble (non-targeted – KD NT) siRNA was used as control. The lentivirus containing these sequences were a kind gift from Prof. Luis Moita (IMM, Portugal), who designed the sequences and constructed the lentiviral vectors. Given these were part of a pre-existent library that did not include siRNAs for the specific knockdown of the fast isoform, we only used the already existent siRNAs.

Infection was performed 24h after plating $3.5x10^4$ cells per well in 96 well plates, by incubating the cells with the lentiviral vectors for 1h30min at 600*g*, 37ºC. Positive knockdowns of MDA-MB-435 were selected with Puromycin after determining the optimal concentration of 0.4 μg/mL. Efficiency of infection was determined by real-time RT-PCR using TaqMan® with primers/probe specific for each of the isoforms, and the Human GAPD Endogenous Control - Applied Biosystems (Foster City, CA, USA).

3.3.3 RNA isolation and real-time RT-PCR

Total cellular RNA was isolated with the RNeasy mini kit (Qiagen – Foster City, CA, USA)**.** RNA was quantified by absorbance at 260 nm, and purity was determined by absorbance at 280 and 310 nm (NanoDrop, ThermoScientific – Waltham , MA, USA). RNA (1 μg) was converted into cDNA using the QuantiTect® RT kit (Qiagen– Foster

City, CA, USA). An aliquot (20 ng) of the cDNA was then amplified in an ABI Prism 7000 real-time RT-PCR unit using the following TaqMan® Gene Expression Assays (Applied Biosystems – Foster City, CA, USA): acid phosphatase 1, soluble (acp1, Hs00962877 m1), acid phosphatase 1 *fast* isoform, soluble (acp1 *fast* isoform, Hs00964348 g1), acid phosphatase 1 *slow* isoform (acp1 *slow* isoform, Hs00246642 m1). Results were normalized to real-time RT-PCR of GAPDH using the Human GAPD Endogenous Control (4333764F Applied Biosystems) and are expressed using the ΔΔCt method.

3.3.4 LMW-PTP enzymatic activity

Enzymatic activity of LMW-PTP was measured in MDA-MB-435 cells as previously described [7,8]. Briefly, lysis buffer, containing 10mM *p*-nitrophenyl phosphate and 0.1% Triton X-100 in 0.1 M sodium acetate, 10mM EDTA, pH 5.5, was added to cells for 2h. Samples were alkalinized by adding 1M NaOH and absorbance of *p*-nitrophenol (PNP) was measured at 405 nm. Results are expressed in mM PNP/min and normalized to total protein content, determined by Precision Red™ Advanced Protein Assay Reagent from Cytoskeleton (Denver, CO, USA).

3.3.5 Proliferation assay

Proliferation rates of both the parental cell line and KDs were determined using PrestoBlue (Invitrogen) assay. 1,650 cells per well were plated in 96 well plates and allowed to adhere for 24h. Proliferation was evaluated after 24h, 72h and 96h by incubation with Presto Blue during 2 hours. Fluorescence was determined with a bottom probe on a fluorescent microplate reader (excitation: 560nm; emission: 590nm, Infinite 200 multimode Reader, Tecan – Mannerdorf, Switzerland). Cell proliferation was confirmed by direct cell counting using a hemacytometer, and the results obtained were not different from the results obtained using PrestoBlue.

3.3.6 Migration assay

Migratory potential of both the parental cell line and KDs were determined using Platypus Technologies Oris™ (Madison, WI, USA) Cell Migration Assay - Collagen I Coated. Briefly, 25,000 cells per well were plated in 96 well plate provided with the assay and allowed to adhere for 24 hours. At that time, stoppers were removed**,** allowing cells to migrate freely. Wells from which the stoppers were not removed (t=0) were taken as controls. After 24 hours, stoppers from the control wells were removed and all wells were immediately stained with Calcein-AM (Calbiochem – Darnstat, Germany) (1mg/mL) for 1h at 37ºC. Fluorescence was determined with a bottom probe on a fluorescent microplate reader (excitation: 485nm; emission: 528nm, Infinite 200 multimode Reader, Tecan), and pictures of wells were taken with a Zeiss (Jena, Germany) Axiovert 200M – Motorized Widefield Fluorescence Microscope.

3.3.7 Invasion Assay

Invasive potential of both the parental cell line and KDs were determined using a 24-well BD BioCoat™ Tumor Invasion System (BD Biosciences – San Jose, CA, USA), following the manufacturer´s instructions. Briefly, 1,650 cells were harvested and plated in the upper chamber, while complete growth medium was added to the lower chamber. The system was incubated for 24 h at 37°C, 5% CO_2. Cells that invaded and migrated through the BD Matrigel Matrix membrane were post-stained with 4 μg/ml of Calcein-AM (Calbiochem - Darnstat, Germany) in Hank's buffered salt solution at 37°C, 5% CO_2 for 1h. Fluorescence was determined with a bottom probe on a fluorescent microplate reader (excitation: 494nm; emission: 517nm, Infinite 200 multimode Reader, Tecan - Mannerdorf, Switzerland).

3.3.8 Rho A activation

RhoA activation of both the parental cell line and KDs were determined using the colorimetric assay RhoA G-LISA™ Activation Assay (Cytoskeleton – Denver, CO, USA. Results were normalized by total protein and not total RhoA protein, according to the manufacturer's instructions.

3.3.9 EphA2 dephosphorylation

Levels of EphA2 dephosphorylation (ratio EphA2 phosphorylated/EphA2 Total) on both the MDA-MB-435 parental cell line and LMW-PTP knockdowns were determined using the ELISA commercial kits Human Total EphA2 DuoSet IC and Human Phospho-EphA2 DuoSet IC (R&D Systems - Minneapolis).

3.3.10 Statistical analysis

All data are expressed as mean ± standard deviation. Significance was established by Student’s t-test or ANOVA and post-hoc Sidak, as appropriate. Differences were considered significant at $p < 0.05$.

The study does not need ethical approval

3.4 Results

3.4.1 LMW-PTP knockdowns

In order to study the influence of LMW-PTP and its isoforms on the migratory and invasive potential of an epithelial tumor cell line, we produced knockdowns of these proteins using siRNA. After screening 6 tumor cell lines [9] we chose MDA-MB-435 as a model of a breast invasive tumor cell line. This choice was based on the differences of endogenous expression between LMW-PTP fast and slow isoforms [9] and the high invasive potential of this cell line.

Efficiency of knockdowns was confirmed by evaluation of mRNA expression and LMW-PTP enzymatic activity.

Figure 3.1 shows mRNA expression levels of the parental cell line and all KDs compared to control (KD NT). Knockdown of total LMW-PTP and the two LMW-PTP isoforms was achieved in 3 clones (CII, CIII and CV) and clone CIV was a specific knockdown of the *slow* isoform.

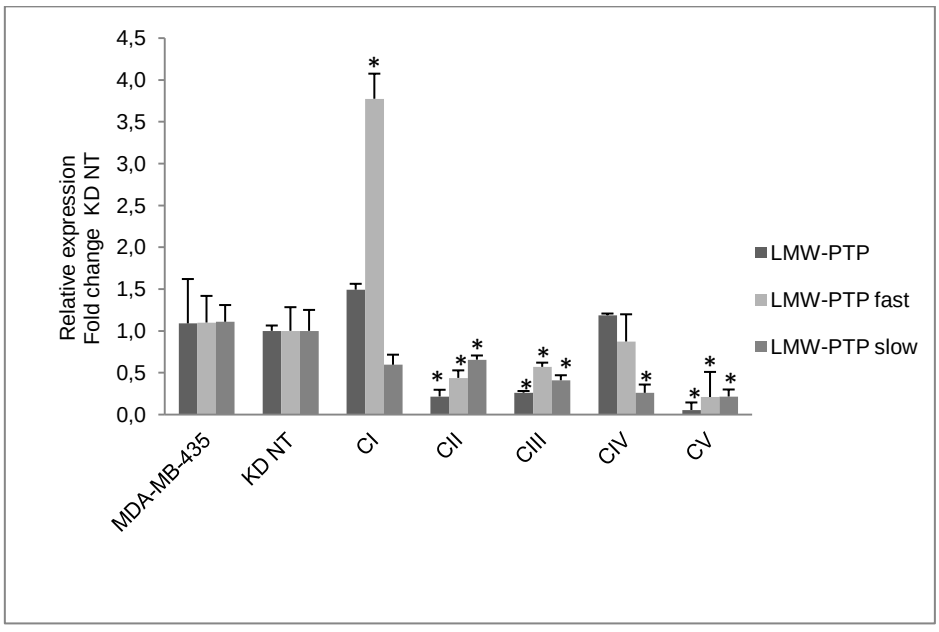

Figure 3.1 - Relative expression of LMW-PTP and its *fast* and *slow* isoforms on the MDA-MB-435 cell line and knockdowns. Error bars represent standard deviation (n=4). *p<0.05 compared to KD NT and MDA-MB-435. KD NT – scramble sequences siRNA (control); CI, CII; CIII; CV – knockdown of total LMW-PTP; CIV – knockdown of LMW-PTP slow isoform.

To confirm these results we determined the LMW-PTP enzymatic activity – Figure 3.2. All five clones showed decreased enzymatic activity compared to KD NT.

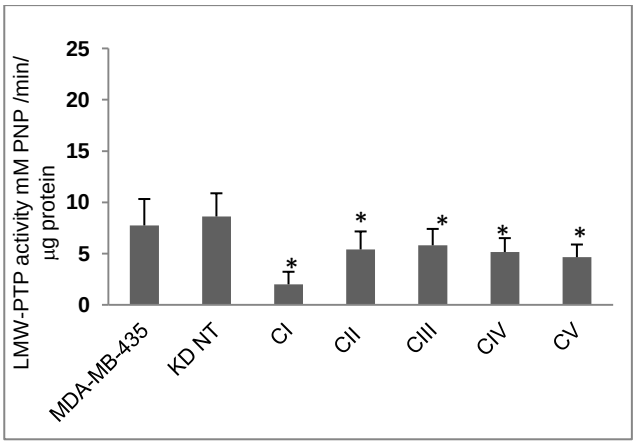

Figure 3.2 - LMW-PTP activity on MDA-MB-435 cell line and knockdowns. Error bars represent standard deviation (n=4). *p<0.05 compared to KD NT and MDA-MB-435. KD NT – scramble sequences siRNA (control); CI, CII; CIII; CV – knockdown of total LMW-PTP; CIV – knockdown of LMW-PTP slow isoform.

Based on these results, we chose two clones for further studies: clone CIV (KD LMW-PTP slow), for being the only clone that showed a specific knockdown of the *slow* isoform, and clone CV (KD LMW-PTP) due to being the clone where the knockdown of LMW-PTP was more effective: 95% compared to 80% of CII and CIII.

3.4.2 Proliferation rate is not altered when LMW-PTP is suppressed

Tumor cells are known to have high rates of proliferation, and it has been reported that the LMW-PTP *slow* isoform causes growth arrest [10]. Therefore, we evaluated how the knockdowns of LMW-PTP could interfere with the growth rate of MDA-MB-435. Results show that knockdowns do not change the growth rate of these cells – Figure 3.3. Cell proliferation was confirmed by direct cell counting using a hemacytometer, and the results obtained were not different from the results obtained using PrestoBlue..

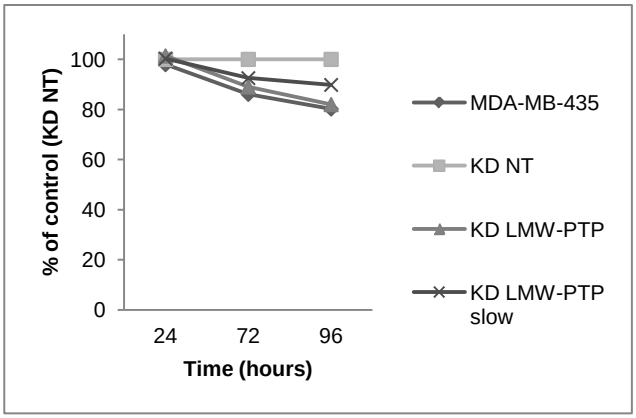

Figure 3.3 - Cell proliferation of different clones compared to KD NT (n=6). $p > 0.05$ for all comparisons. KD NT – scramble sequences siRNA (control); KD LMW-PTP – total LMW-PTP knockdown; KD LMW-PTP slow – LMW-PTP slow isoform knockdown.

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3.4.3 Suppression of LMW-PTP induces MDA-MB-435 migration

Migratory potential is important for cancer cells to spread and colonize different organs, thereby initiating the metastatic process. We evaluated if the LMW-PTP knockdowns changed the migratory potential of this cells. Both KD LMW-PTP *slow* and KD LMW-PTP migrate more than KD NT and the parental cell line Figure 3.4.

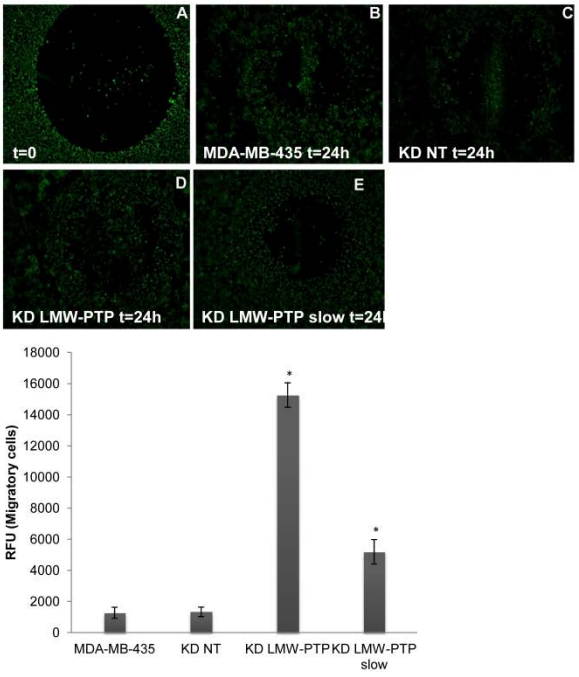

Figure 3.4- A-E Representative images of cell migration assay. A: t=0, before migration; B-E: t=24h after migration. B: MDA-MB-435; C: KD NT; D: KD LMW-PTP; E: KD LMW-PTP *slow* isoform.
Cell migration of LMW-PTP KDs and MDA-MB-435. Error bars represent standard deviation (n=3). *$p<0.05$ compared to KD NT and MDA-MB-435. KD NT – scramble sequences siRNA (control); KD LMW-PTP – total LMW-PTP knockdown; KD LMW-PTP slow – LMW-PTP slow isoform knockdown.

3.4.4 Suppression of LMW-PTP does not change MDA-MB-435 invasion potential

To determine how the suppression of LMW-PTP could influence the invasive potential of MDA-MB-435 cells, we also evaluated the invasive potential of these clones. Analysis of the invasive potential in a Matrigel matrix showed that there were no differences between any of the KD LMW-PTP and MDA-MB-435 (Figure 3.5).

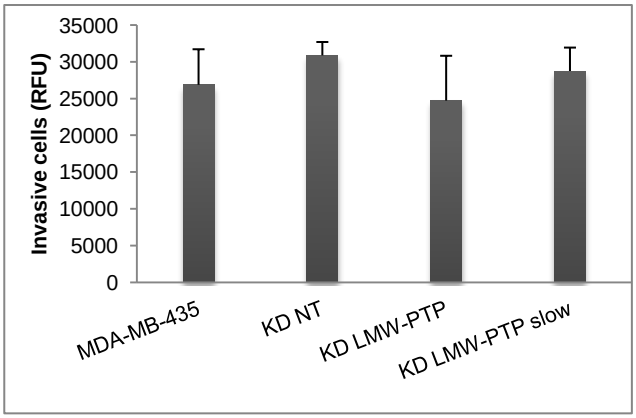

Figure 3.5 - Cell Invasion of LMW-PTP KDs and MDA-MB-435. Error bars represent standard deviation (n=3). $p > 0.05$ for all comparisons. KD NT – scramble sequences siRNA (control); KD LMW-PTP – total LMW-PTP knockdown; KD LMW-PTP slow – LMW-PTP slow isoform knockdown.

3.4.5 Rho A activation is decreased when LMW-PTP is suppressed

To further understand the mechanism by which LMW-PTP can affect the migratory potential, we evaluated the activation status of RhoA. RhoA is an important molecule that regulates cellular adhesion and migration. Results show that RhoA activation is altered in the two knockdowns (Figure 3.6): KD LMW-PTP had a decreased RhoA activation , whilst KD LMW-PTP *slow* had an increased RhoA activation.

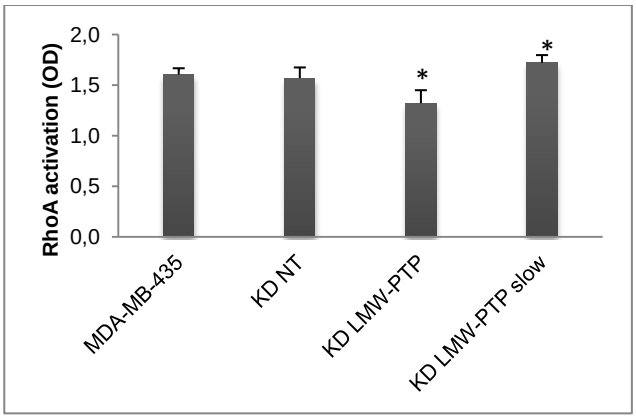

Figure 3.6 - RhoA activation in LMW-PTP KDs and MDA-MB-435. Error bars represent standard deviation (n=3). *$p<0.05$ compared to KD NT and MDA-MB-435. KD NT – scramble sequences siRNA (control); KD LMW-PTP – total LMW-PTP knockdown; KD LMW-PTP slow – LMW-PTP slow isoform knockdown.

3.4.6 Phosphorylation status of EphA2 is not altered when LMW-PTP is suppressed

Given the EphA2 receptor can be a substrate for LMW-PTP, we determined if knockdowns of LMW-PTP influenced the phosphorylation levels of this receptor. Analysis of Figure 3.7 shows that when LMW-PTP is suppressed the phosphorylation status of EphA2 is not changed; neither on the KD LMW-PTP nor on the KD LMW-PTP *slow*, suggesting that, in this model, LMW-PTP does not influence EphA2 phosphorylation status.

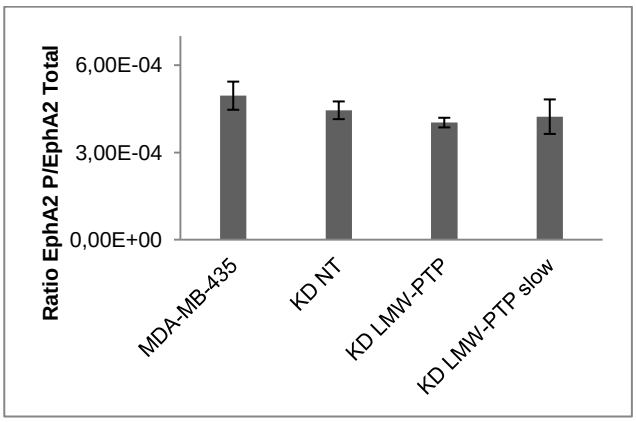

Figure 3.7 - Dephosphorylated EphA2 in LMW-PTP KDs and MDA-MB-435. Error bars represent standard deviation (n=3). $p > 0.05$ for all comparisons. KD NT – scramble sequences siRNA (control); KD LMW-PTP – total LMW-PTP knockdown; KD LMW-PTP slow – LMW-PTP slow isoform knockdown.

3.5 Discussion

The role of LMW-PTP in tumorigenesis has been controversial. As a phosphatase, it is associated with growth arrest through blocking of signal transduction elicited by kinases, thus being considered an oncosuppressor; however, it has been found to be overexpressed in different types of tumors [11] and associated with poor prognosis [12].
In this study, we examined the effect of LMW-PTP suppression in MDA-MB-435, an aggressive breast cancer cell line. Using 5 siRNAs, we successfully suppressed total LMW-PTP and its *slow* isoform.
Confirmation of LMW-PTP isoforms' knockdown effectiveness can only be achieved through specific mRNA expression, since protein quantification by western blot analysis

is not possible because there are no available antibodies targeting LMW-PTP isoforms. Also, enzymatic activity of the two isoforms can not be determined separately. However, LMW-PTP activity is differentially associated with its isoforms: it is known that the *slow* isoform contributes the most to LMW-PTP activity, also showing a higher enzymatic activity than the *fast* isoform [13]. Accordingly, and given all KDs showed knockdown of the *slow* isoform, all clones had a lower enzymatic activity than KD NT. The exception is the CI. Cells that were infected with lentivirus containing this target siRNA sequence showed a different behaviour of the isoforms. Due to these inconsistent and unexpected results we did not proceed our studies with these clones.

Enhanced proliferation is one of the main characteristics of tumors. The LMW-PTP *slow* isoform can be involved in cell proliferation via two pathways: PDGF-R and EphA2. The relationship between PDGF-R and LMW-PTP has been shown in the NIH3T3 cell line, but there are no previous evidences of this interaction in tumor cell lines. In NIH3T3, PGDF-R is a substrate of the LMW-PTP *slow* isoform. Through dephosphorylation of this receptor, the LMW-PTP *slow* isoform blocks PDGF-induced signalling, decreasing cell growth [10]. However, our results show no differences in the proliferation rates of the KDs. We hypothesize that, given MDA-MB-435 has a high proliferation rate, with a population doubling time of 22h, this is probably not under the sole control of PDGF signalling, and hence cannot be changed only by the absence of LMW-PTP.

LMW-PTP is also known to dephosphorylate EphA2, increasing tumor cell growth and differentiation [14], being the control of the tumorigenic potential of LMW-PTP associated with EphA2 phosphorylation status. EphA2 is dephosphorylated in a wide range of cancer cells and this phenomena seems to correlate with malignancy: tumor cell growth, survival, migration and invasion [15]. Our results show that suppression of LMW-PTP did not change the phosphorylation status of EphA2, suggesting that, in MDA-MB-435 cells, LMW-PTP is not the main regulator of EphA2 phosphorylation status. The tumorigenic potential of these cells may be so dependent on EphA2 phosphorylation that LMW-PTP *per se* is not sufficient to revert this phenotype. Also, the absence of differences in EphA2 phosphorylation status, between all KDs and control, may explain the same growth rate of all KDs and the control, further supporting our hypothesis. All studies that associate LMW-PTP with dephosphorylation of EphA2 have been performed in transformed normal cells, such as MCF10A, a mammary

epithelial cell line: EphA2 overexpression is sufficient to cause tumorigenesis in MCF10A cells. However, others have shown that a dominant negative of LMW-PTP did not change EphA2 phosphorylation level significantly, suggesting that EphA2 may not be a major substrate of LMW-PTP in MCF10A [6], and this may also be the case in MDA-MB-435.

On the other hand, LMW-PTP can also be regulated by EphA2 [6]. These authors showed that this regulation is not direct, proposing Src as an intermediary between EphA2 and LMW-PTP. The proposed mechanism, in MCF10A cells, is that EphA2 overexpression probably promotes destabilization of the adherens junctions through a signalling pathway of recruitment of Src kinase, enhanced LMW-PTP activity, inhibition of p190RhoGAP and activation of RhoAGTPase [6].

Based on this model, we evaluated the activation levels of RhoAGTPase in the KDs. Our results show that KD LMW-PTP decreased RhoA activation, which should be due to suppression of the *fast* isoform, whilst KD LMW-PTP *slow* activated RhoA. RhoA activation is controlled by GEFs (guanosine nucleotide exchange factors) and GAPs (GTPase activating proteins). One important GAP is p190RhoGAP. This protein is involved in cytoskeleton rearrangement and seems to be one of LMW-PTP cytoskeletal *(fast)* associated fraction specific substrates [3]. Thus, supression of LMW-PTP *fast* will cause p190RhoGAP activation with the consequent inactivation of RhoA. As for the effect of the *slow* isoform on RhoA, there are no reports concerning this isoform's ability to interact with p190RhoGAP. We may hypothesize, based in other authors' results [16, 17] that the interaction between the LMW-PTP slow isoform and p190RhoGap may occur through Src kinase: knockdown of the LMW-PTP slow isoform could inactivate Src kinase [16], which will cause p190RhoGAP inactivation and consequently RhoA activation [17].

Our results show that the LMW-PTP *slow* isoform has the opposite role of the *fast* isoform regarding RhoA activation. Regardless of RhoA increased or decreased activation the effect on the migratory potential of cells is the same: both KD LMW-PTP and KD LMW-PTP *slow* cause an increase in the migration potential of MDA-MB-435, with the former having a higher migratory potential ($p<0.05$). These apparently controversial results may be explained by previous reports showing that regulation of cell-cell adhesion and, consequently, cell migration, can be achieved through the balance between RhoA GTP/RhoA GDP [18]. Therefore, we hypothesize that the larger

increased migration potential of the KD LMW-PTP is mainly due to the lack of the *fast* isoform, suggesting that the *fast* isoform may be more important for the metastatic process than the *slow* isoform, since migration is a characteristic of metastatic tumor cells.

After migration, metastatic cells have to be able to invade surrounding tissues to colonize distante organs. Although it is known that LMW-PTP interacts with β-catenin and E-cadherin [19], molecules that regulate epithelial-mesenchymal transition and consequently invasion, LMW-PTP knockdowns had no effect on the invasive potential, leading us to suggest that in MDA-MB-435 cells the invasive potential is not under control of LMW-PTP.

3.6 Conclusions

Taken together, our results show that regulation of LMW-PTP expression and activity, with the consequent balancing of RhoA activation, affects the migratory potential of these cells. This effect is more pronounced in the KD LMW-PTP, suggesting that the fast isoform has a more important role in cell migration and can thus be more prominent in tumor progression than in tumor growth, whilst the slow isoform may be important on an earlier stage of the tumorigenic process.

The fast and slow isoforms seem to have opposite roles in RhoA activation, although leading to the same final effect of increased migratory potential. Apparently, both the increase or decrease of RhoA activation will have the same effect on cell migration, suggesting that any deregulation of RhoA, regardless of being activation or inhibition, will affect cell migration. Therefore, regulation of LMW-PTP is an important feature for cancer cell migration.

Finally, we propose that new therapeutic approaches may be considered using not only total LMW-PTP but targeting specifically its two main isoforms, fast and slow.

3.7 Acknowledgments

The authors wish to thank Dr. Luis Moita from Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Portugal for his kind gift of lentivirus vectors with siRNA sequences targeting LMW-PTP. Ricardo Pires for technical support.

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Chapter 4

Low molecular weight protein tyrosine phosphatase slow isoform knockdown in MDA-MB-435 cells decreases RAW 264.7 osteoclastic differentiation

4 Low molecular weight protein tyrosine phosphatese slow isoform knockdown in MDA-MB-435 cells decreases RAW 264.7 osteoclastic differentiation

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4.1 Abstract

Bone metastases from breast cancer are a common characteristic of advanced tumors and cause significant patient morbidity and mortality. During the bone metastatic process, tumor cells and bone cells drive a vicious cycle with the different players such as tumor cells, osteoblasts and osteoclasts, stimulating growth and activity of each other. IL-6 and IL-8 are two important factors released by tumor cells that regulate osteoclastic activity.

Low molecular weight protein tyrosine phosphatase (LMW-PTP) is a polymorphic enzyme with two main isoforms, *fast* and *slow*, that has been associated with bone metabolism and cancer. LMW-PTP is also involved in the regulation of Src activity. We evaluated the effect of soluble factors released by the MDA-MB-435 breast cancer cell line in RAW 264.7 osteoclastogenesis. We show that these soluble factors do not change RAW 264.7 osteoclastogenic potential. However, through siRNA targeting of LMW-PTP and LMW-PTP *slow* isoform in MDA-MB-435 cells, the KD (knockdown) of the LMW-PTP *slow* isoform decreased osteoclastogenesis of RAW 264.7, evaluated by TRAP staining and quantification. The LMW-PTP *slow* isoform KD also showed a less active Src. We also show that the knockdown of LMW-PTP and its slow isoform decreases the release of IL-8 but not IL-6 by MDA-MB-435.

Thus, we have shown that the LMW-PTP *slow* isoform can be an important protein in bone metastatic disease, with a relevant role in the interplay between tumor cells and osteoclasts through the regulation of Src activity and IL-8 secretion. Further studies exploring the pathway LMW-PTP-Src. should be performed in order to unravel the role of the different molecules involved in this pathway as new prognostic markers and possible novel therapeutic approaches.

4.2 Introduction

Breast cancer ranks among the most prevalent malignancy in women. Breast carcinoma frequently metastasizes to bone, and approximately 70% of patients develop bone metastases The process of metastasis, including the spread and growth of tumor cells in distant organs, is paramount to the definition of malignancy – once a tumor metastasizes to bone, it is usually incurable (1).

The development of bone metastases is associated with numerous debilitating skeletal-related events (SREs) – pathological fractures, hypercalcemia, radiotherapy and surgery to bone (1). SREs are associated with a considerable decrease of patients' quality of life, and increased morbidity and mortality (2).

Breast cancer cells in bone do not directly resorb bone but can regulate the activity of osteoblasts and consequently osteoclasts causing disruption of bone remodeling. (3). Bone derived growth factors will stimulate metastatic cancer cells, further promoting tumor growth in bone, driving the vicious cycle of bone metastases (4).

Patients with bone metastases are treated with anti-tumor drugs targeting tumor cells and with anti-resorptive drugs, such as bisphosphonates, that inhibit osteoclastic bone resorption. Among patients undergoing bisphosphonates therapy 25% do not respond to treatment (5, 6), but the mechanism underlying this therapeutic differential response is currently unknown. Therefore, it is important to address this question and understand which molecules are involved in this process.

Low-molecular weight protein tyrosine phosphatase (LMW-PTP), a polymorphic enzyme widely expressed in different tissues, has been considered as an important signaling molecule in osteoblast biology and bone metabolism (7). Regarding tumorigenesis, LMW-PTP has recently been associated with different types of cancers (8), such as breast and lung. The two main isoforms of LMW-PTP, *fast* and *slow*, seem

to have different roles in the development of breast tumor(9), with the *fast* genotypes having been positively associated with cancer (10, 11). LMW-PTP has also been shown to be involved in the regulation of Src activity (7), an important protein for osteoclastic activity.

Given the above, we hypothesized that LMW-PTP may be involved in bone metastatic disease and its isoforms may have a differential role in the communication between tumor cells and osteoclasts. Since the soluble factors produced by human or mouse breast cancer cells, such as IL-6 (12) and IL-8 (13), can directly stimulate osteoclast differentiation from late human or mouse osteoclast precursors (14), we tested how a specific knockdown of LMW-PTP and its *slow* isoform in the MDA-MB-435 breast carcinoma cell line (9) can influence the differentiation of RAW 264.7 murine monocytic cells in osteoclasts. Our results show that the specific knockdown of the LMW-PTP *slow* isoform decreases the potential of osteoclastic differentiation of RAW 264.7 cells probably due to the decrease of Src activation and lower levels of IL-8 produced by this KD.

4.3 Materials and Methods

4.3.1 Cell culture

MDA-MB-435, a breast cancer epithelial cell line, was obtained from ATCC (American Type Culture Collection), cultured in DMEM with 10% fetal bovine serum (FBS) and 1% pennicilin/streptomycin and the conditioned medium (CM) was harvested when cells reached 80% confluence.

RAW 264.7 cells, murine monocyte cells for osteoclast differentiation, were kindly provided by Dr. Michael Rogers (Garvan Institute, Sydney, Australia). These cells were maintained in DMEM, 10% heat inactivated FBS and 1% penicillin/streptomycin. For the differentiation in osteoclasts, cells were plated in 96 well plates, 950 cells/well in *a*-MEM with 10% heat inactivated FBS, 1% penicillin/streptomycin and RANKL 100ng/ml. On day three cells were supplemented RANKL 100 ng/ml and either fresh

medium or 60% CM , and cultured for another 2 days. Supernatants were collected on day 5 for TRAP quantification and cells were stained for TRAP.

4.3.2 LMW-PTP knockdowns

Five different siRNA sequences were designed to specifically knockdown the total and the *slow* isoform (KD LMW-PTP slow) One scramble (non-targeted – KD NT) siRNA was used as control. The lentivirus containing these sequences were a kind gift from Prof. Luis Moita (IMM, Portugal), who designed the sequences and constructed the lentiviral vectors. Infection and clone selection were performed as described in chapter 3 (accepted for publication in *PLOS ONE*).

4.3.3 TRAP staining

Cells were stained for TRAP using the commercial kit Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma) without the final step with acid hematoxilin. Osteoclasts were identified as multinucleated TRAP positive cells, with more than three nuclei.

4.3.4 TRAP quantification

TRAP quantification was performed in the supernatant of cells using the commercial kit MouseTRAP^{TM} Assay (TRACP ELISA´s – ids) , a solid phase immunofixed enzyme activity assay for the determination of osteoclast derived tartarate-resistant acid phosphatase form 5b (TRACP 5b) in mouse serum. Results are expressed in U/L TRAP.

4.3.5 Src phosphorylation

Src activation status was determined by Western blot analysis. MDA-MB-435 cells were seeded in 6 well plates and allowed to grow. At approximately 80% confluence cells were washed once with PBS, lysed in 200 µl 2x SDS-loading buffer with protease and phosphatase inhibitors cocktails (Sigma-Aldrich), and heated to 95ºC for 10 min. Samples were loaded onto a 10% polyacrylamide gel and electrophoresis was performed using a Mini-PROTEAN Tetra cell (BioRad). Proteins were transferred onto a Protran BA85 nitrocellulose membrane (Whatman) using a Mini-PROTEAN Tetra Cell transfer system (BioRad). Membranes were blocked in PBST, 5% nonfat dry milk for 1 h, incubated overnight with the primary antibody and for 1h with the secondary antibody. Antibody detection was performed using SuperSignal West Pico Chemiluminescent HRP Substrate (Pierce) according to the manufacturer's instructions and signal was visualized on radiographic film. Antibodies against total Src (#2108), phospho Y_{527}Src (#2107), non-phospho Y_{416}Src (#2101), and anti-rabbit, anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technologies. Total Src was used as loading control.

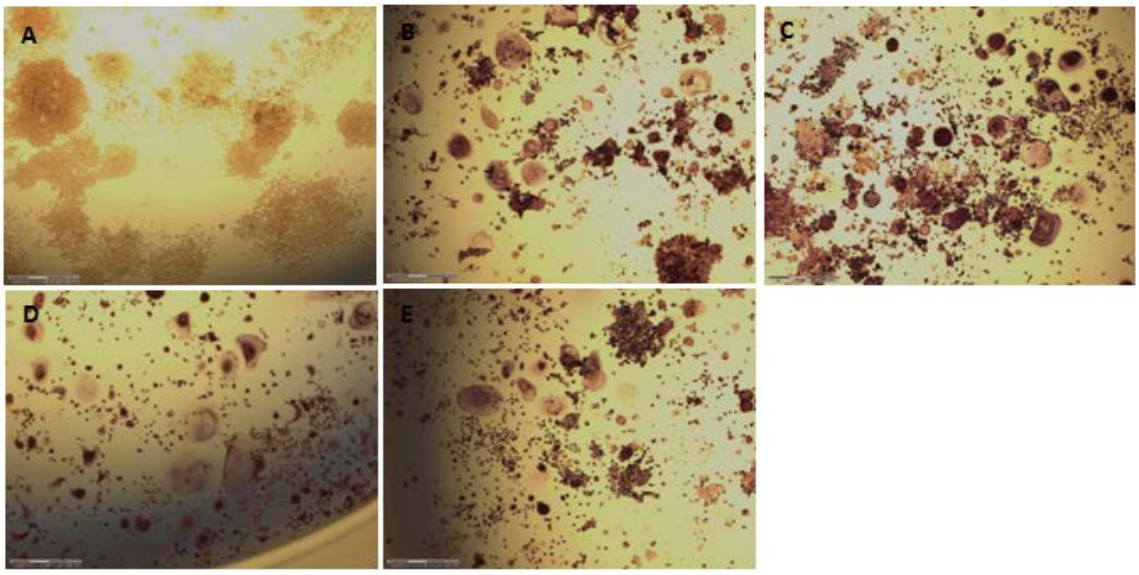
4.3.6 IL-6 and IL-8 quantification

IL-6 and IL-8 were measured in cell culture supernatants. Briefly, supernatants from MDA-MB-435, KD NT, KD LMW-PTP and KD LMW-PTP *slow* at approximately 80% confluence were harvested, centrifuged at 200g, 5 min and assayed immediately using a commercial ELISA kit for IL-6 and IL-8 quantification: Human IL-6 Quantikine HS ELISA kit and Human IL-8/CXCL8 Quantikine ELISA kit – R&D systems, respectively. Results were expressed in pg/mL.

4.4 Results

4.4.1 Conditioned medium from MDA-MB-435 breast cancer cell line does not change osteoclastogenesis in RAW 264.7 cell line

Osteoclastogenesis was assessed using RAW 264.7 cells. In the absence of RANKL 100ng/mL, RAW cells did not differentiate into osteoclasts (negative control, Figure 4.1 A and Figure 4.1 F). In contrast, supplementation with RANKL 100ng/ml (positive control, Figure 4.1 B and Figure 4.1F) induced osteoclastogenesis. MDA-MB-435 CM did not change osteclastogenesis compared to the positive control –Figure 4.1 C and Figure 4.1 F. Since there were no differences between MDA-MB-435 (parental cell line) and KD NT, our results in KD will be compared with the parental cell line.



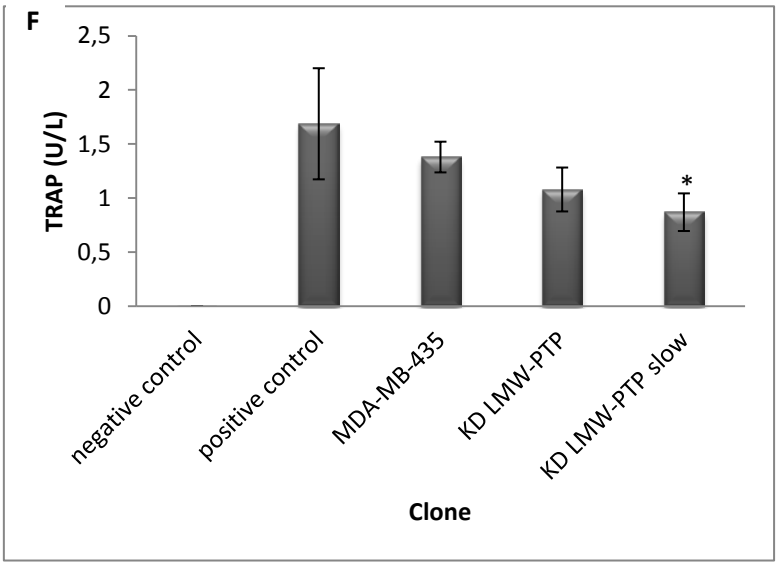


Figure 4.1 Osteoclast differentiation depending on conditioned medium. RAW 264.7 cells were stimulated with RANKL (100ng/ml), stained for TRAP and TRAP was quantified in cells supernatants as described in the section "Materials and Methods". **A.** negative control: differentiation medium without RANKL; **B-F.** representative images of osteoclasts derived from RAW 264.7 cells in different differentiation media; **B.** positive control: differentiation medium with 100ng/ml RANKL; **C-E.** osteoclasts differentiation in conditioned medium derived from tumor cells: **C.** MDA-MB-435; **D.** MDA-MB-435 KD LMW-PTP; **E.** MDA-MB-435 KD *slow* isoform. **F.** TRAP quantification in cells supernatants. Data are mean ± standard deviation from 3 independent experiments. * $p<0.05$ compared with differentiation with conditioned medium from MDA-MB-435

4.4.2 LMW-PTP slow isoform knockdown decreases the ability of breast cancer-derived factors to induce osteoclastogenesis

Comparison between the differentiation of RAW 264.7 cells in the presence of CM from different MDA-MB-435 knockdowns showed that only the KD LMW-PTP *slow* decreased osteoclastogenesis –Figure 4.1 A and Figure 4.1 F.

4.4.3 LMW-PTP slow isoform knockdown decreases activated Src in MDA-MB-435

Src is only fully activated when Tyr 527 is dephosphorylated and Tyr 416 phosphorylated. The increase of Tyr 527 phosphorylation and Tyr 416 dephosphorylation in the KD LMW-PTP *slow* compared with MDA-MB-435 (Figure 4.2) represents a decrease in Src activation whereas the total Src – loading control - was not changed.

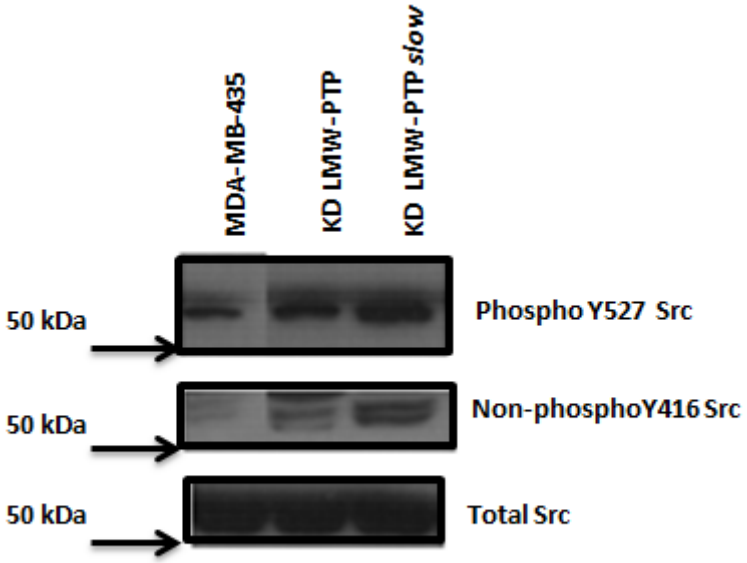


Figure 4.2 Phosphorylation of Src. Total Src was used as loading control.

4.4.4 LMW-PTP knockdown decreases IL-8 secretion but not IL-6 by MDA-MB-435

Secretion of IL-6 and IL-8 by tumor cells in bone are crucial for the maintenance of the vicious cycle of bone metastasis through osteoclasts stimulation. Our results showed that LMW-PTP knockdown in MDA-MB-435 decreased the secretion of IL-8 (Figure 4.3) but not IL-6 (Figure 4.4).

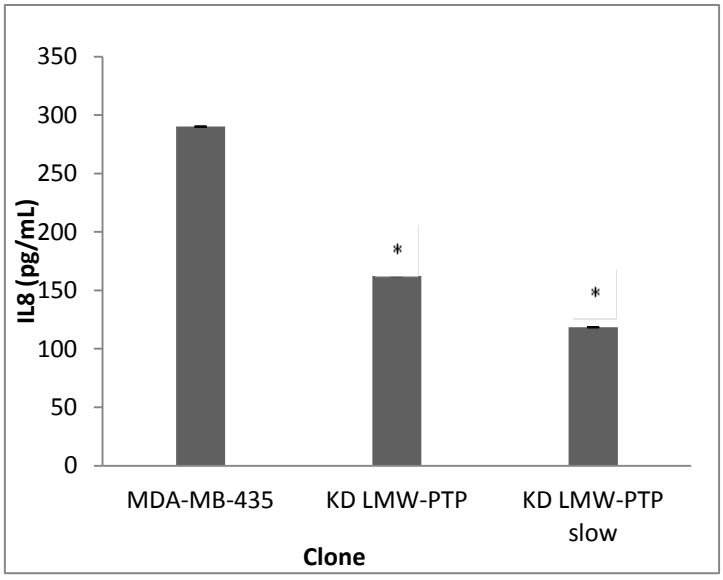


Figure 4.3 LMW-PTP knockdown in MDA-MB-435 decrease IL-8 secretion by these cells

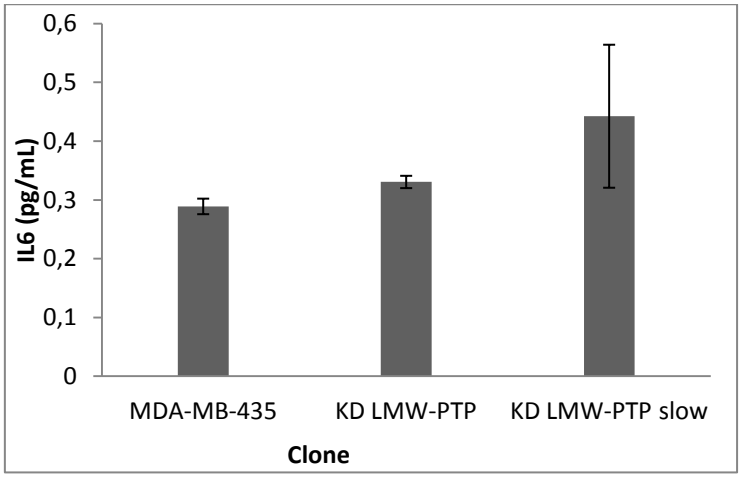


Figure 4.4– LMW-PTP knockdown did not change IL-6 secretion by MDA-MB-435

4.5 Discussion

The understanding of the interplay between cancer cells and bone cells is crucial to reveal new mechanisms and new targets in the so called vicious cycle of bone metastastic disease. In this work, it was our aim to address how LMW-PTP isoforms influence the cross talk between cancer cells and osteoclasts during this process.

Our results show that, in the absence of RANKL, factors released by MDA-MB-435 do not stimulate differentiation of RAW 264.7 into osteoclasts. However, suppression of the LMW-PTP *slow* isoform in MDA-MB-435 decreased the osteoclastogenic potential of RAW 264.47 even in the presence of RANKL. LMW-PTP has been associated with the control of Src activity, and Src also controls LMW-PTP activity (7, 15, 16). Therefore, in order to explore further how this phosphatase can be involved in the regulation of osteoclastogenesis, we evaluate the activity of Src in MDA-MB-435 and the release of IL-6 and IL-8 by these cells, since these cytokines are secreted by breast cancer cells in bone and are known to modulate osteoclastic activity (12).

Src activity is controlled by two tyrosine residues, Y527 and Y146. Src is activated when Y527 is non-phosphorylated and Y416 is phosphorylated (16). Our results show that in LMW-PTP *slow* KD, Src is less active than in the MDA-MB-435 parental cell line. Src has an important role in physiological and pathological processes such as cell proliferation and tumorigenesis (17). Recently, Ferreira et al (15) showed that knockdown of LMW-PTP reverts chemoresistence in a chemoresistant cell line, Lucena-1, due to a decrease in Src activation. This is in accordance with our results, since Src is more inactive in the LMW-PTP *slow* isoform KD.

A mechanism by which Src inactivation is important for tumor cell invasion may be the interaction between this protein and MMPs (16). It is known that, in the vicious cycle of bone metastases, MMPs are important proteins in the cross-talk between tumor and bone cells (4). We may speculate that, by regulating Src, LMW-PTP can have the ability to interact with MMPs, being an important factor in bone metastases development. Src has also been described as a predictive factor for bone metastization. Based on bioinformatics, Zhang et al (18) discovered a strong association between late-onset bone metastasis and tumor Scr activity in a cohort of over 600 breast cancer patients. Src supports cancer cell survival in the bone marrow microenvironment (18). Thus, and given our results showed a decrease in Src activation when the LMW-PTP *slow* isoform is knockdowned, the findings by Zhang et al could explain the decrease of osteoclastogenesis in the RAW 264.7 cell line exposed to the conditioned medium of MDA-MB-435 with the LMW-PTP slow isoform KD.

Regarding IL-8 results, Bendre et al described IL-8 as a potent direct activator of osteoclastic differentiation independently of RANKL, involving the IL-8 receptor (CXCR1) on the surface of osteoclasts and their precursors (13, 19). Our results show

that LMW-PTP KDs secrete lower levels of IL-8 than the MDA-MB-435 parental cell line. Therefore, LMW-PTP could interfere with the production of IL-8 and thus decrease osteoclastogenesis in RAW 264.7 cells exposed to conditioned medium from MDA-MB-435 with the LMW-PTP *slow* isoform knocked-down. The fact that only the *slow* isoform suppression decreased osteoclastogenesis suggests that: 1) the *slow* isoform has the ability to change the secretion of IL-8 but 2) other factors involved in osteoclasts' differentiation are being changed by the *fast* isoform, thereby explaining that although the secretion of IL-8 is decreased in the two knockdowns, only the conditioned medium from MDA-MB-435 *slow* KD decreased the differentiation of osteoclasts. Furthermore, our results suggest that the role on LMW-PTP in the vicious cycle of bone metastasis is independent of osteoblasts, since these cells are not present in our experimental system.

IL-6 is less expressed in MDA-MB-435 than IL8 (20) and some authors describe that MDA-MB-435 does not produce IL-6, only MDA-MB-231 (21). Our results show that although the concentration of IL-6 in the supernatant of MDA-MB-435 cells is much lower than IL-8, this cytokine is also secreted by this breast cancer cell line, though independently of LMW-PTP expression. Therefore, we conclude that alterations in osteoclastogenesis caused by the LMW-PTP *slow* isoform KD in MDA-MB-435 are independent of IL-6 secretion.

In a recent study we proposed that, in breast cancer cell lines, the LMW-PTP *slow* isoform may have an oncogenic role and the *fast* isoform an opposite, anti-oncogenic role (9). However, the role of the two isoforms in tumor progression has not been addressed. Based on our previous findings associating LMW-PTP with tumor cells migration (accepted for publication in *PLOS ONE*- chapter 3), both LMW-PTP isoforms seem to be involved in tumor progression. Taken together, these results may indicate that the *slow* isoform not only is involved in tumorigenesis (9) and tumor cells migration (accepted for publication in *PLOS ONE* – chapter 3) but also increases the affinity of MDA-MB-435 towards bone, which suggests that the *slow* isoform may be responsible for the increased interaction between breast cancer cells and osteoclasts. Given there are no studies regarding LMW-PTP isoforms and tumor progression in bone, further studies are needed to address this question *in vivo*.

Taken together, our results show that the expression of the LMW-PTP *slow* isoform in tumor cells may be a prognostic marker for tumors in bone – increased expression of the

LMW-PTP *slow* isoform may be a marker for a high propensity for tumors to metastasize to bone. Further research on the interaction between LMW-PTP, Src, IL-8 and MMPs could provide new approaches for developing novel drugs for bone metastatic disease.

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Chapter 5

Low molecular weight protein tyrosine phosphatase isoform are differentially expressed in the different stages of tumor progression

5 Low molecular weight protein tyrosine phosphatase isoform are differentially expressed in the different stages of tumor progression

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5.1 Abstract

Protein tyrosine phosphorylation is a post-translational modification that is fundamental for cellular functions like growth, adhesion and migration. Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) are the enzymes implicated in this reversible process. Given this modification in so crucial for such vital processes, the deregulation of PTKs and PTPs can be important in human pathologies such as cancer. LMW-PTP is a tyrosine phosphatase that has been associated with breast and lung cancer. The aim of this study was to access if LMW-PTP isoforms are differentially expressed in normal tissue, primary breast cancer and bone metastatic breast cancer tissue. Using real-time RT-PCR analysis we evaluated the expression levels of total LMW-PTP and its two main isoforms, *fast* and *slow*, in human samples of normal breast, primary breast cancer tissue and bone metastatic breast cancer tissue (n: 5, 4 and 7, respectively). LMW-PTP total mRNA expression was – increased in primary breast cancer tissue and *fast* isoform expression was increased in metastatic tissue. The s*low* isoform mRNA expression was slightly increased in primary and metastatic tissue, although without reaching statistical significance. Our findings suggest that the expression of LMW-PTP isoforms, namely the *fast* isoform, changes during tumor progression, being this isoform only up-regulated in metastatic tissue.

5.2 Introduction

Protein tyrosine phosphorylation is a post-translational modification crucial for cell signaling metabolism. Sequential phosphorylation/dephosphorylation reactions are governed by the action of protein tyrosine kinases (PTKs) and phosphatases (PTPs), respectively. This reversible phosphorylation control is fundamental for physiological functions such as cell growth, cell cycle, metabolism and cytoskeletal function. The deregulated activity of PTPs is, therefore, involved in numerous human diseases, namely cancer(1).

Almost 107 different genes are described as coding for PTPs. Among these genes, 81 are predicted to be active protein phosphatases (2). According to the primary structure of the catalytic reaction, PTPs are divided in four classes.

Low-molecular weight protein tyrosine phosphatase (LMW-PTP), a class II cys-based PTP, is polymorphic and widely expressed, with no particular tissue-specific expression. This enzyme has two main isoforms, derived from alternative splicing of a single transcript: IF1/*fast* isoform and IF2/*slow* isoform. Although the two isoforms only differ by a sequence of 42 amino acids, their functions are markedly different: the *slow* isoform, in the cytosolic pool, associates with the growth factor receptor like PDGF-R and EphA2, dephosphorylating membrane receptors. On the other hand, the *fast* isoform, in the cytoskeleton pool, dephosphorylates cytoskeleton proteins such as p190RhoGap, important for cellular mobility (3).

Recently, different studies have associated LMW-PTP with cancer (1, 4-7). However, the roles of the two main isoforms in this pathology have not been described, and the importance of this enzyme in cancer remains controversial. Previous studies suggest the involvement of LMW-PTP in tumorigenesis, but studies characterizing LMW-PTP isoforms in human cancer samples are lacking. The aim of the present work was to determine the expression levels of LMW-PTP mRNA in breast carcinomas – primary breast tumor tissue and metastatic breast tumor tissue. Normal breast tissue was used as control. Our results show that the expression of the two main LMW-PTP isoforms is variable in the different stages of tumor progression, which suggests that the fast and slow isoforms have different roles in this process.

5.3 Materials and Methods

5.3.1 Ethics statement

Studies involving human samples were performed in accordance to the principles of the Declaration of Helsinki, approved by the Ethical Committee of Santa Maria Hospital – CHLN, and all patients signed an informed consent.

5.3.2 Clinical samples

Five normal breast tissue (NBT) and four tissue samples from primary breast cancer (PBCT) were collected from mastectomy specimens. Seven tissue samples from bone metastases from breast cancer (MBCT) were obtained as part of standard of care surgical treatments of patients with pathologic fracture of spinal cord compression due to metastatic disease. The samples are not paired samples.

5.3.3 RNA extraction

RNA extraction was performed as described by Casimiro et al (8). All surgical specimens were embedded in OCT compound (Sakura) and snapshot frozen in liquid nitrogen within 30 min of collection. For tumor cells microdissection, 12 μm cryostat sections were transferred to pre-cooled polyethylene napthalate (PEN) membrane slides (Carl Zeiss MicroImaging GmbH) and stained with cresyl violet. Briefly, slides were kept at -80ºC until usage; air dried for 30 s; fixed in ice-cold 70% ethanol for 2 min; washed in ice-cold RNase-free dH_2O for 1 min; stained in 1% cresyl violet acetate for 1 min; and dehydrated by 1 min immersion in 70% ethanol followed by 100% ethanol. Slides were air dried and immediately used for laser microdissection and pressure catapulting (LMPC) using a Laser PALM – Microbeam 4.2 microdissection system (Carl Zeiss MicroImaging GmbH). A total area of 500,000 μm^2 corresponding to tumor cells was microdissected for each sample. Total RNA was extracted using RNeasy Plus

Micro Kit (Quiagen). Total RNA concentration and quality was determined with the RNA 6000 Pico kit (Agilent) (9).

5.3.4 Real-time RT-PCR

Total RNA (8 μl) was reverse transcribed using RT^2 Nano PreAmp cDNA Synthesis kit (SABiosciences, Quiagen). cDNA was then amplified in an ABI Prism 7500 fast real-time RT-PCR unit using the following TaqMan® Gene Expression Assays (Applied Biosystem): acid phosphatase 1, soluble (acp1, Hs00962877 m1), acid phosphatase 1 *fast* isoform, soluble (acp1 *fast* isoform, Hs00964348 g1), acid phosphatase 1 *slow* isoform (acp1 *slow* isoform, Hs00246642 m1). Results were normalized to real-time RT-PCR of GAPDH using the Human GAPD Endogenous Control (4333764F Applied Biosystems) and are expressed using the ΔΔCt method.

5.3.5 Statistical analysis

All data are expressed as mean ± standard deviation. Significance was established by Student's t-test or ANOVA and post-hoc Sidak, as appropriate. Differences were considered significant at $p < 0.05$.

5.4 Results

The levels of total LMW-PTP mRNA and mRNA for the *fast* and *slow* isoforms were evaluated by real-time PCR analysis. Comparison between primary breast cancer tissue, metastatic breast cancer tissue and normal breast tissue shows that LMW-PTP mRNA levels are variable depending on tumor progression (Figure 5.1).

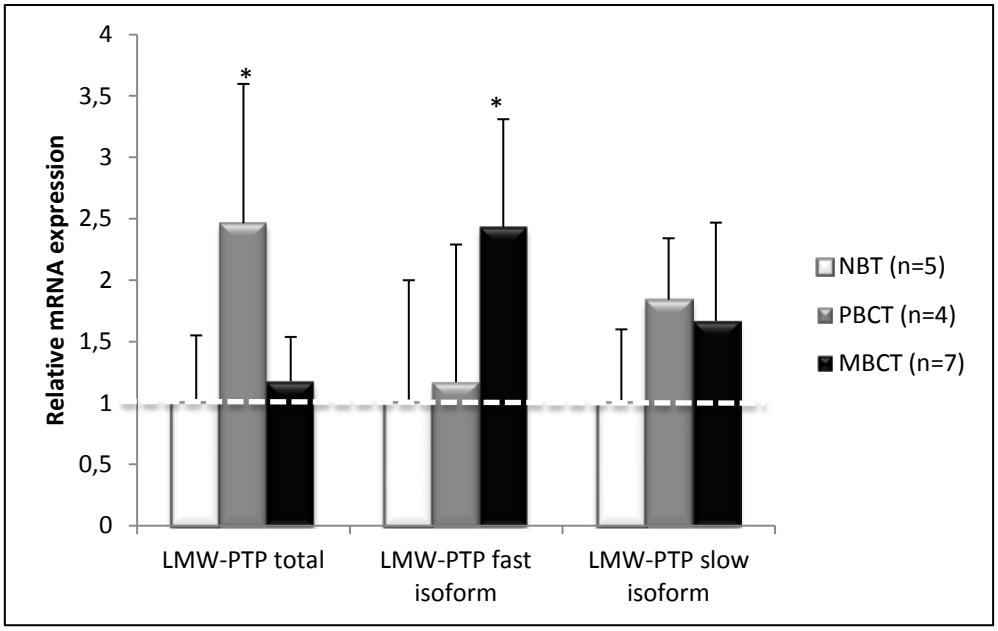

Figure 5.1 – LMW-PTP mRNA relative expression in different stages of tumor progression. Error bars represent standard deviation**.** *p<0.05 compared to normal breast tissue;

5.5 Discussion

The association of LMW-PTP with cancer has been largely discussed. As a phosphatase, by dephosphorylation of substrates that are involved in cell growth, it was initially described as having anti-oncogenic potential, regulating negatively neoplastic transformation.

However, in cell culture models, both an anti- and pro-oncogenic role of LMW-PTP has been described. The anti-oncogenic role is described by its association with PDGF-R(10), FGF-R(11), insulin receptor (12), FAK (13) and STAT (14, 15), whilst the pro-oncogenic role is due to its association with EphA2 (16), EphB1 (17), p190RhoGAP (18), β-catenin (19) and JAK (20).

Only one study reports the importance of LMW-PTP in human surgical samples. Malentacchi et al (5) described an increase in total LMW-PTP mRNA expression in colon and breast cancer compared to adjacent normal tissue, with no difference in the expression pattern of the two main LMW-PTP isoform between normal and tumor tissue (5).The authors conclude that LMW-PTP has an oncogenic role.

Our results show an increase of total mRNA expression in breast primary tumors compared with normal breast tissue suggesting an oncogenic potential for total LMW-

PTP. Regarding the isoforms, the *slow* isoform expression shows a slight, but not significant, increase.

Regarding MBCT, there is an increase of the *fast* isoform expression both compared with normal tissue and primary tumors. Total LMW-PTP in MBCT shows the same expression pattern as normal breast tissue. The *slow* isoform has the same relative expression in primary and metastatic tissue.

These results lead us to hypothesize that the two main isoforms of LMW-PTP have different roles in tumor behavior and tumor progression. The increase of total LMW-PTP in primary tumors is in accordance with Malentacchi's results (5) in breast and colon cancers.

As for MBCT, the increase of the *fast* isoform compared to NBT and PBCT suggests that this isoform is the most important for tumor progression, regardless of the expression of the total protein. Given the *fast* isoform is associated with the cytoskeleton, it is more involved in migration and invasion, cellular characteristics that are associated with tumor progression.

Considering possible new pharmaceutical targets, we suggest they should take into account the different stages of tumor development: total LMW-PTP can be more relevant in an early stage of tumor growth , while in tumor progression the *fast* isoform can be considered more important.

Finally, our results suggest that the controversial studies associating LMW-PTP and cancer may be due to the paucity of information regarding the isoforms and the tumor stage.

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Chapter 6

ACP1 polymorphim is not a therapeutic marker or a prognostic marker in patients with bone metastatic disease

6 ACP1 polymorphism is not a therapeutic marker or a prognostic marker in patients with bone metastatic disease

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6.1 Abstract

Patients with bone metastatic disease are commonly treated with anti-resorptive drugs, such as bisphosphonates, in addition to anti-tumor drugs. However, 25% of these patients remain with uncontrolled levels of bone resorption despite of therapy..ACP1/LMW-PTP is a protein tyrosine phosphatase that is widely expressed and could be involved in bone metabolism through the regulation of Src activity. Our aim was to evaluate how the ACP1 *fast/slow* polymorphism could condition patients' differential response to anti-resortive drugs. We studied 144 patients with bone metastasis. ACP1 polymorphism was evaluated by PCR-RFLP and therapeutic response was evaluated by monthly assessment of urinary NTX. NTX was determined by an ELISA commercial kit and the results were normalized to urinary creatinine levels. Our results show that the ACP1 *fast/slow* polymorphism does not determine the response to bisphosphonates therapy in patients with bone metastasis. Moreover, this polymorphism does not affect the number of SREs, skeletal morbidity rate, radiographic pattern of bone lesions, time to SREs, time to progression and overall survival. Therefore, the ACP1 *fast/slow* polymorphism can not be used either as therapeutic response marker or as prognostic marker.

6.2 Introduction

Metastases to bone in breast cancer patients occur in up to 80% of patients with advanced disease (1). Tumor cells in bone result in bone resorption acceleration, leading to skeletal related events (SREs), such as pathological fractures, requirement of radiotherapy to bone, hypercalcemia, need for orthopedic surgery and spinal cord compression.

The stimulation of osteoclast function by tumor cells in bone is of particular importance, resulting in osteolysis, which is typically associated with the normal coupling between osteoblasts and osteoclasts (2). The increase of osteoclastic activity is the rationale for the use of bisphosphonates as antiresortive drugs in the management of metastatic disease. Therefore, patients with bone metastatic disease are treated with anti-tumor and anti-resorptive drugs.

Bisphosphonates are a class of drugs that inhibit the mevalonate pathway, specifically farnesil pyrophosphate (FPP) synthase, blocking protein prenylation. Protein prenylation is a post-translational modification of GTP-binding proteins, such as Ras and Rho (2), being an essential feature for osteoclastic activity. The effect of bisphosphonates on bone cell function influence serum and urinary levels of biochemical markers of bone metabolism, and thus these markers can be used to monitor the progress of the disease and response to treatment. Monitoring of bone metastatic disease is largely limited to radiographs and isotope scans (1). However, the current use of imaging methodologies limit the detection of patients' bone health before the development of symptoms (3). There is now growing evidence that bone turnover marker measurements may complement imaging methods by allowing a more rapid and quantitative evaluation and aiding therapeutic management decisions at an early stage (1).

The dynamics of bone tissue, by the action of osteoblasts and osteoclasts, is associated with the release of distinct biochemical markers, that can be measured in serum, such as ICTP (type I collagen C-terminal telopeptide) and BALP (bone-specific alkaline phosphatase), or in urine, such as NTX (N-telopeptide of type I collagen) and CTX (C-telopeptide of type I collagen). The use of this type of markers provides a relatively non-invasive means to assess ongoing bone turnover as a whole (3).

Among patients undergoing bisphosphonates therapy, around 25% do not normalize bone resorption markers (3), and these have worst prognosis: the overall survival is decreased, and both the number of SREs and bone metastatic disease progression events are increased. The causes for that are not understood, and the individual genetic variability could interfere in this process.

Low molecular weight protein tyrosine phosphatase (LMW-PTP) is an enzyme that is associated with bone metabolism, namely regarding osteoblasts (4). LMW-PTP is a polymorphic enzyme, coded by the *ACP1* (acid phosphatase locus 1) gene, with six possible genotypes (AA, AB, AC, BB, BC, CC) that have strong differences in the enzymatic activity. LMW-PTP has two different isoforms, named *fast* and *slow* according to their electrophoretic mobility. *ACP1* genotypes can be grouped in *fast* or *slow* depending on the relative expression of the fast or slow isoforms: "*fast* genotypes": BB,AB; "*slow* genotypes": AA, AC, BB, BC,CC (5, 6).

Based on previous studies regarding *ACP1* polymorphism and cancer, and since there are no studies associating the *ACP1* polymorphism with therapeutic response in patients with bone metastatic disease, we evaluated if this polymorphism could contribute to the genetic variability that may be responsible for the differences found in the response to bisphosphonates therapy.

6.3 Patients and methods

6.3.1 Patients and study design

One-hundred and forty-four consecutive cancer patients with documented radiological evidence of advanced bone disease were included in this study. Patients were evaluated for the presence of bone metastases at baseline and followed up prospectively with an objective evaluation of metastatic disease performed every 3 or 4 months. At baseline, all patients with documented bone metastases were classified according to x-ray pattern (lytic, blastic or mixed). The number and timing of SREs was also analyzed during the study. Skeletal morbidity-rate is the ratio of the number of skeletal complications to the time unit. Disease progression was defined as the appearance of new bone lesions.

Bisphosphonate therapy consisted of two possible regimens: pamidronate 90mg intravenously monthly or zoledronate 4mg in 100ml 0,9% NaCl, intravenously over 15minutes, every three or four weeks.
The study protocol was approved by the Ethics Committee of Hospital Santa Maria – Centro Hospitalar de Lisboa Norte. All patients provided written informed consent.

6.3.2 NTX levels determination

Urinary NTX was determined by Osteomark NTx Urine ELISA (Inverness). The detection limit of the NTX assay is 20 nM BCE (bone collagen equivalents units – assay value, does not include creatinine excretion). The urinary levels of NTX in bone collagen equivalent units are expressed as the ratio to urine creatinine excretion. Urinary NTX was defined as low (NTX<50 nmol BCE/mmol creatinine) or high (NTX ≥ 100 BCE nmol/mmol creatinine). The cutoff values for NTX were chosen to reflect the approximate upper limit of normal (ULN). However, the normal range for urinary NTX varies according to age, gender and endocrine function. The ULN in young healthy adults is approximately 50 nmol/mmol creatinine. After menopause in women and during androgen deprivation therapy in men, the ULN levels is approximately 100 nmol BCE/mmol creatinine (7). Therefore, analysis based on both 50 and 100 nmol BCE/mmol creatinine was performed.

6.3.3 Genetic polymorphism identification

ACP1 polymorphism was analyzed by PCR-RFLP as described by Alho et al (8).

6.3.4 Statistical analysis

Descriptive statistics were performed comparing baseline characteristics between NTX phenotypes. Univariate analysis assessing the effect of ACP1 phenotype in NTX normalization at 3 and 6 months, SREs, skeletal morbidity rate and radiographic pattern of bone lesions was conducted using regression analysis. Further multivariate models

were performed. Results are presents as odds ratios (OR) for logistic regression or beta coefficient for linear regression with 95% confidence intervals (CI).

The effect of ACP1 phenotype in time to event variables as overall survival, time to bone disease progression and time to skeletal related events was assessed using univariate and multivariate Cox proportional hazards models. Results are presented as hazards ratios (HR) with CI 95%.

6.4 Results

Of the 144 cancer patients included in the study with a mean age of 59.1±13.8 years, 66.7% were females. 89 (61.8%) patients had breast cancer, 25 (17.4%) had prostate cancer and 30 had other types of primary tumors. There was a median follow up time of 67months. ACP1 *fast* genotypes were the most common (64.8%). 84.7% of the patients had the NTX levels higher than normal at the time of bone disease diagnosis (Table 6.1). Results of 144 cancer patients are presented because data were analyzed separately and aggregated, with the same final results.

Normalization of NTX levels (<50 nmol BCE/mmol creatinine) in patients grouped according to their ACP1 genotypes was analyzed at 3 and 6 months after initiating bisphosphonates therapy. There were no statistical differences regarding the normalization rate of NTX between *fast* and *slow* patients (Table 6.2).

The number of SREs, skeletal morbidity rate and radiographic pattern (type of bone lesion) was not statistically different between *fast* and *slow* patients (Table 6.4).

Analysis of the overall survival, time to bone disease progression and time to skeletal related event, showed that ACP1 genotypes did not influence any of these parameters. (Table 6.4 and Figure 6.1)

Table 6.1 – Population characteristics

Parameter	Population sample	ACP1 *slow*	ACP1 *fast*
Number of pts, (%)	144 (100%)	50 (35.2%)	92 (64.8%)
Patients gender, n (%)			
Female	96 (66.7%)	27 (54%)	68 (73.9%)
Male	48 (33.3%)	23 (46%)	24 (26.1%)
Age in years at dx, mean ± SD	59.1 ± 13.8	61.3 ± 12.7	57.7 ± 14.1
Follow up in months, median [IQR]	67.2 [27,41-117,7]	68.3 [36,92-120,03]	62.1 [20,95-109,18]
Cancer type, n (%)			
Breast	89 (61.8%)	26 (52%)	62 (67.4%)
Prostate	25 (17.4%)	13 (26%)	11 (12%)
Other	30 (20.8%)	11 (22%)	19 (20.7%)
Elevated NTX (≥ 50) at bone disease diagnosis, n (%)			
All cancer	122 (84,7%)	40 (80%)	81 (88%)
Breast cancer	76 (85.4%)	21 (80.8%)	55 (88.7%)

Table 6.2- NTX normalization (<50 nmol BCE/mmol creatinine) at 3 and 6 months post BP therapy

NTX normalization (<50) at 3 and 6 months post BP therapy	Sample	ACP1 *slow*	ACP1 *fast*	
3 months post BP therapy, n (%)	38 (41.3%)	16 (55.17%)	22 (34.9%)	OR=0,67 IC95% [0,31-1,43]; p=0,3
6 months post BP therapy, n (%)	40 (55.6%)	17 (65.4%)	23 (50%)	OR=0,65 IC95% [0,31-1,27] p=0,26

Odds ratio – Odds Ratio, IC95% - confidence interval 95%. ACP1 codification: *fast* 1 *slow* 0

Table 6.3– SREs, skeletal morbidity rate and radiographic pattern of bone lesions

SREs, skeletal morbidity rate and radiographic pattern of bone lesions	**Sample**	**ACP1** ***slow***	**ACP1** ***fast***	
SREs post BP therapy introduction, n (%)	71 (49.3%)	24 (48%)	45 (48.9%)	OR=0,95 IC95% [0,52-1,73] p=0,743
Skeletal morbidity rate, median	0.047	0.064	0.041	Beta=-0.17 IC95% [-0,41-0,07] p=0,16
Radiographic pattern of bone lesions, n (%)				OR=1,29 IC95% [0,56-2,97] p=0,55
Lytic	78 (58.7%)	29 (59.2%)	48 (58.5%)	
Blastic	32 (24.1%)	14 (28.6%)	18 (22%)	
Mixed	23 (17.3%)	6 (12.2)	16 (19.5%)	

OR – Odds Ratio, IC95% - confidence interval 95% ACP1 codification: *fast* 1 ; *slow* 0

Table 6.4– Overall survival, time to bone disease progression and time to skeletal related event

Overall survival, time to bone disease progression and time to skeletal related event	**Log- rank test (Univariable analysis)**
Overall survival	HR=1,096 IC95% [0,73-1,65] p=0,66
Time to bone disease progression	HR=0,95 IC95% [0,52-1,72] p=0,86
Time to skeletal related event	HR=1,09 IC95% [0,64-1,87] p=0,743

HR – Hazard Ratio, IC95% - confidence interval 95%

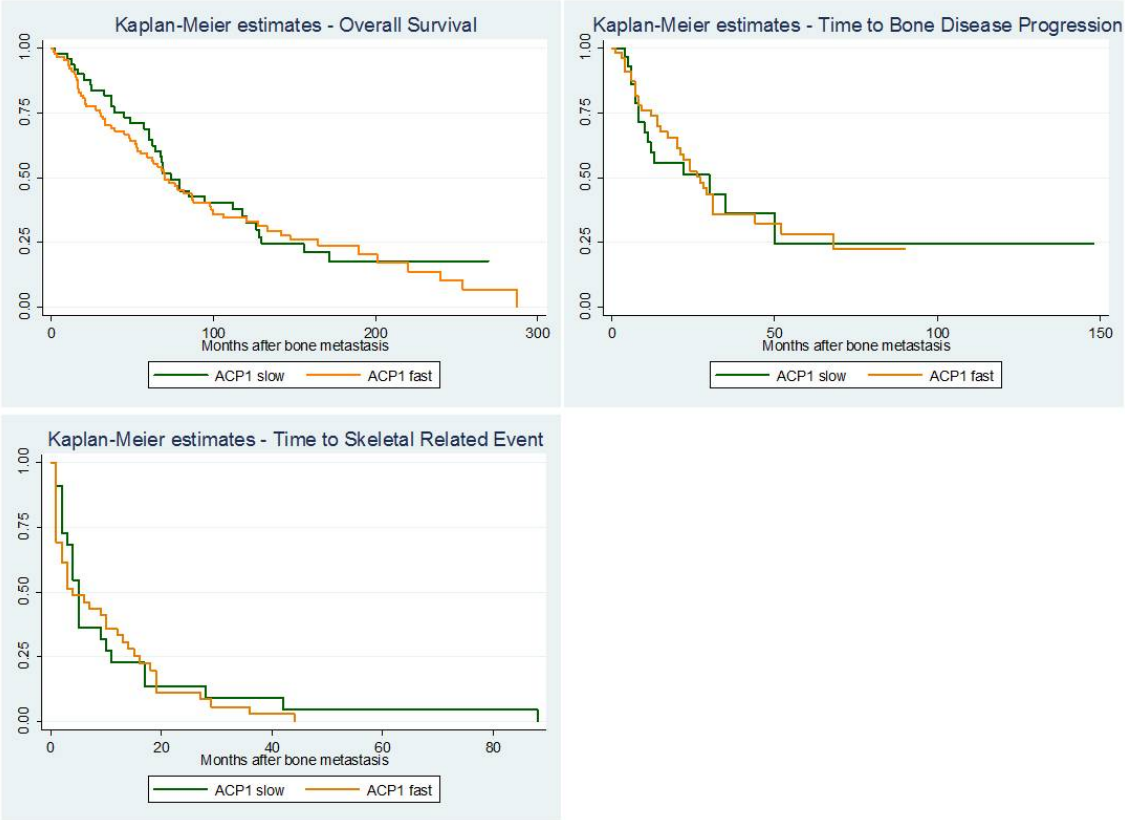

Figure 6.1- Kaplan Meier estimates of A- overall survival; B- time to bone disease progression and C- time to skeletal related event, separated by *fast* and *slow* ACP1 genotypes.

6.5 Discussion

Bone metastatic disease disrupts the balance between bone resorption and formation and in many circumstances favors the increase of bone resorption. The crosstalk between tumor cells in bone and bone cells, namely osteoblasts and osteoclasts, is the main feature of bone metastization. The activity of these three main players of metastatic disease to bone can be measured by some biochemical markers such as NTX and BAP. NTX is released as a consequence of osteolysis. The measurement of this bone marker in urine, using a monoclonal antibody, should be normalized with a renal function marker – creatinine - to control the hydration status and renal function (3). Different studies in patients with bone metastasis (1, 9) describe NTX levels as a specific marker for bone disease, better than conventional tumor markers in predicting progression of metastatic disease in bone. Also, the levels of NTX decrease with bisphosphonates therapy (9). Therefore, NTX could be a valid bone resorption marker to predict bone

metastases progression in patients with or without bisphosphonates therapy. NTX levels have the highest diagnostic accuracy for bone metastases status when compared to other bone markers (BAP and ICTP) (9).

The fact that 25% of the patients with bone metastatic disease, treated with bisphosphonates, do not normalize NTX levels is intriguing. One of the explanations could be based on the genetic variability of each patient. To address this question, we evaluated if the *ACP1* fast/slow polymorphism could contribute to this differential response to treatment.

The strong differences in enzymatic activity between ACP1 *fast* and *slow* genotypes suggest possible effects at the clinical level, for susceptibility, development or progression of tumors (10). Two different studies defend an association between the *ACP1* polymorphism and cancer (5, 11), and the pattern observed is similar in these two studies. Alho et al (5) found a predisposition of carriers with "*fast* genotypes" to advanced cancer and Spina et el report a protective effect of the slow isoform concerning colon cancer development (11).

Our results suggest that *ACP1* genotypes do not seem to be implicated in NTX normalization. Moreover, no significant differences were found regarding SREs, skeletal morbidity rate, radiographic pattern of bone lesions, overall survival, time to bone disease progression and time to skeletal related events between the two patients groups: ACP1 *fast* and ACP1 *slow*.

Therefore, although LMW-PTP isoforms could be important intumor progression , as described previously (Chapter 5) , and tumor progression, they don´t seem to be useful as prognostic markers in patients with bone metastases.

Therefore, LMW-PTP and its isoforms seem to be important in the tumor itself (from our previous studies) but not in the host response to the tumor and to therapy. Also, grouping the patients according to *ACP1* genotypes is an extrapolation of the relative expression of the *fast* and *slow* isoforms, which may be insufficient to draw conclusions. To properly address this question, further studies are needed. A possible way to solve this question can be through the evaluation of the mRNA expression of the different isoforms in primary and metastatic tissue and correlate it with the different outcomes.

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Chapter 7

The role of low molecular weight protein tyrosine phosphatase (LMW-PTP ACP1) in oncogenesis - review paper

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7 The role of Low Molecular weight protein tyrosine phosphatase (LMW-PTP ACP1) in oncogenesis

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REVIEW

The role of low-molecular-weight protein tyrosine phosphatase (LMW-PTP ACP1) in oncogenesis

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Abstract Protein tyrosine phosphorylation is a crucial cellular event that is involved in the most important processes of cellular metabolism. Low-molecular-weight protein tyrosine phosphatase (LMW-PTP) is a tyrosine phosphatase that presents two active distinct isoforms and is regulated through cysteine oxidation and tyrosine phosphorylation. This enzyme has been linked to tumorigenesis, but its role is considered controversial: it may be considered oncogenic or anti-oncogenic depending on its interaction with different substrates. Furthermore, recent studies have demonstrated that LMW-PTP is involved in epithelial cell migration, a characteristic of tumor cells. This fact strengthens the importance of this enzyme in the oncogenic process and opens new avenues for future research. The study of LMW-PTP and its pathways may enhance therapeutic strategies that target tyrosine phosphorylation and its substrates. In this review, we try to clarify the importance of this protein in carcinogenesis through the analysis of LMW-PTP interaction with different substrates.

Keywords Low-molecular-weight protein tyrosine phosphatase · Isoforms · Cancer · Metastasis

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Introduction

Tyrosine phosphorylation is a key event on the communication between and within cells, cell shape and motility, decisions to proliferate versus to differentiate, cellular processes such as regulation of gene transcription, mRNA processing, and transport of molecules in or out of cells [1]. The control of protein tyrosine phosphorylation in vivo is regulated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). A growing body of evidence indicates that the contribution of PTPs to control cell phosphorylation state is as relevant as that of PTKs [1]. These proteins can control other diverse processes such as focal adhesion dynamics and cell-cell adhesion, being themselves regulated by dimerization, phosphorylation, and reversible oxidation [2].

Protein tyrosine phosphatases

The family of PTPs, almost 107 enzymes [1], comprises four classes, established based on the amino acid sequence of their catalytic domain [1], each with a range of substrate specificities: the classical receptor PTPs, the classical non-receptor PTPs, the dual specificity PTPs, and the low-molecular-weight PTPs (class II cysteine-based PTPs). The most significant trait of the protein tyrosine phosphatase superfamily is conservation of the CX_5R signature motif, which forms the phosphate binding loop in the active site (known as the P-loop or PTP-loop) [3].

There is recent evidence that members of the PTP family are key components of tumorigenesis in various human cancers, exerting either putative oncogenic or tumor suppressive function, depending on the cellular context [4]. Among the currently identified 107 PTPs, at least 37 have been implicated in human cancer, with approximately equal proportions of oncogenic and tumor suppressor activities [5]. Sastry and Elferink [6] reviewed the interplay of

receptor tyrosine kinases and protein tyrosine phosphatases in cancer progression, focusing on PTP1B, PTPN11, and PTPN12, defending that these three proteins can have different roles in cancer: the first has a dual role, and the other two have opposite roles, tumor promotion and tumor suppression, respectively.

ACP1-low-molecular-weight protein tyrosine phosphatase (LMW-PTP) has been described as a negative regulator of cellular proliferation induced by growth factors. However, recently, LMW-PTP has been suggested as a positive regulator of tumor onset and progression in animal models [7] and has been shown to increase EphA2 receptor dephosphorylation, which is associated with several human cancers [8, 9].

In this review, we will focus on *ACP1*-LMW-PTP since it seems to be an important enzyme for tumor development and progression and because of its controversial role in the tumorigenic process.

ACP1

The *ACP1* gene is the only human member of the family of class II cysteine-based PTPs [1] and encodes the LMW-PTP (Enzyme Commission no. EC 3.1.3.2), a group of 18-kDa proteins with no particular tissue specificity expression [10]. The *ACP1* gene, located on 2p25.3, is composed of seven exons and six introns (Fig. 1) spanning about 18 kb of genomic DNA [11, 12]. The human *ACP1* is genetically polymorphic, having three alleles, namely, A, B, and C, that give rise to six genotypes—AA, AB, AC, BB, BC, and CC [12, 13]. The gene has 13 transcripts encoding 5 proteins. Of the remaining eight transcripts, four are thought to undergo nonsense mediated decay, a process that prevents the expression of truncated or erroneous proteins, two do not contain an open reading frame, and two contain only intronic sequences [14]. Two of the five translated proteins correspond to the main active isoforms of ACP1, ACP1_001 (NM_004300.3; *fast*) and ACP1_002 (NM_007099.3; *slow*). These two electrophorectically, kinetically, and immunologically distinct isoforms, termed fast and slow based on their electrophoretic mobility [11], arise from mutually exclusive alternative splicing of exon 3 or 4 of the primary transcript (Fig. 1). These two exons, 3 and 4, encode the sequence for the amino acid residues in positions 39–76 for both the fast and slow isoforms. The remaining exons, 1–2 and 5–7, are identical for the two isoforms, encoding the amino acid residues 1–38 and 77–157, respectively [11, 15].

The difference in the expression ratios of these isoforms seems to account for the phenotypic differences. The ratio of their activity differs markedly among genotypes, with fast B, the fast isoform produced by the B allele, being much more prominent than its slow counterpart (ratio 4/1), whereas the slow C isoform exhibits a much higher activity than the ACP1_001 fast C (ratio 1/4). The two A isoforms, fast A and slow A, occur in the ratio 2/1 [16].

These isoforms seem to be associated with different cell compartments. For many years, LMW-PTP was reported as an exclusively cytosolic enzyme, but Cirri et al. [17] demonstrated that LMW-PTP exists in two spatially and functionally separate pools: the fast isoform is associated to the cytoskeleton fraction, whereas the slow isoform exists in the cytosolic pool.

The activity of LMW-PTP is regulated by tyrosine phosphorylation/dephosphorylation and reversible oxidation of cysteine residues.

LMW-PTP activity: phosphorylation and redox regulation

The regulation of LMW-PTP activity occurs in two major mechanisms: tyrosine phosphorylation and redox regulation.

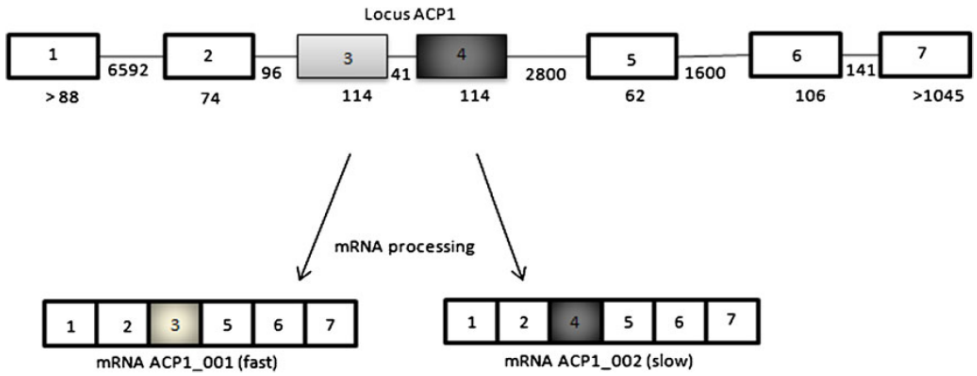

Fig. 1 Gene structure of the human ACP1 locus. Mechanism for the generation of fast and slow isoforms by mutually exclusive RNA splicing. Adapted from Rudbeck [15]

LMW-PTP contains two conserved adjacent tyrosines, Tyr131 and Tyr132, which are the preferential sites of phosphorylation by PTKs, mainly Src kinases [5, 18, 19]. Src tyrosine kinase strongly phosphorylates both Tyr131 and Tyr132. The phosphorylation of these two tyrosines is independent and results in different outcomes [20]: when the phosphorylation occurs on Tyr131, the activity of LMW-PTP increases 25-fold, whereas the phosphorylation of Tyr132 does not affect enzymatic activity [20]. Phosphorylation of Tyr131 leads to regulation of Rho-mediated cell adhesion rate through dephosphorylation of p190RhoGAP [20]. On the other hand, phosphorylation of Tyr132 leads to an in vitro Grb2 (growth factor receptor-bound protein 2) binding [19]. Therefore, although enzymatic activity does not seem to change, this could lead to an increase of the strength of cell adhesion through down-regulation of MMP expression, probably through the inhibition of the Grb2/MAPK pathway [20]. Thus, Chiarugi et al. [20] proposed that LMW-PTP is a bifunctional phosphatase that regulates both the strength and the rate of cell adhesion through alternative phosphorylation of Tyr131 or Tyr132.

LMW-PTP is inactivated by transient chemical modification of an essential cysteine (Cys 12) present at the active site, which forms a disulfide bond with a proximal cysteine (Cys 17), also within the active site loop. This oxidation process is reversible and therefore regulates the activity of the enzyme [21, 22]. The reversibility of oxidation and the consequent recovery of enzymatic activity are generally recognized as key points in the redox regulation of this protein. This redox regulation of LMW-PTP may be used by cancer cells to survive and proliferate, as proposed by Souza et al. [4]. These authors suggested that the two forms of LMW-PTP, active (reduced) or inactive (oxidized), play important roles in cancer cell signaling: the reduced form can be involved in transformation, via EphA2 dephosphorylation, and loss of adhesion, by inhibition of p190RhoGAP; the oxidized form can promote survival pathways through activation of JAK2 and STAT5. Thus, cancer cells can benefit from LMW-PTP redox regulation to thrive [4].

LMW-PTP is therefore a redox sensor that can act synergistically with redox regulation of Src. Src tyrosine kinase is activated through oxidation (formation of a disulfide bond), dephosphorylation of Tyr527, and phosphorylation Tyr416 [23]. Reactive oxygen species (ROS) generated through nicotinamide adenine dinucleotide phosphatase (NADPH) complex can activate Src and inhibit LMW-PTP. On the other hand, when Src is fully activated, it associates with LMW-PTP and induces tyrosine phosphorylation and activation of the phosphatase, which, in turn, can promote Src inactivation. This positive-feedback loop suggests that LMW-PTP is a good candidate for Src inactivation [23]. In fact, Zambuzzi et al. [24] have shown that overexpression of LMW-PTP decreased Src activity in response to PDGF [24].

We propose that the loop between LMW-PTP and Src is one of the most important processes by which LMW-PTP can be considered an important target for cancer therapy. The fact that Src inhibitors are currently used in cancer research supports our hypothesis: Src activity will depend on the different phosphorylation and redox status of LMW-PTP, which, in turn, will favor or preclude cancer cell survival and proliferation.

LMW-PTP interaction with cancer-associated molecules

Protein phosphorylation plays key roles in many physiological processes and is often deregulated in pathological conditions [25] such as cancer, which may be considered a pathology of deregulated signal transduction. The relevant molecules that are apparently regulated by LMW-PTP in cancer progression are the platelet-derived growth factor receptor (PDGF-R) [26], p190RhoGAP [8, 27], ephrin A2 receptor (EphA2) [8, 9], β-catenin [28], focal adhesion kinase (FAK) [29, 30] janus kinase (JAK) [31], and signal transducer and activator of transcription (STAT) [32, 33] (Table 1). The interactions of LMW-PTP with these substrates are discussed below.

PDGF-R

In 1995, Chiarugi et al. [26] suggested that LMW-PTP tightly interacts with activated PDGF-R. This interaction is apparently achieved via its catalytic site, acting on phosphotyrosine residues of the receptor's cytoplasmic domain, leading to receptor inactivation and thus reducing the mitogenic signal. This hypothesis is consistent with the effects of PDGF stimulation in transfected cells, either by overexpression or knock-down of the protein [26].

Other studies with PDGF stimulation have shown that while the LMW-PTP cytosolic pool binds to, and dephosphorylates, activated PDGF-R, the cytoskeleton-associated pool becomes phosphorylated by c-Src during PDGF stimulation and specifically acts on substrates that become tyrosine phosphorylated upon PDGF treatment [10, 17].

Fiaschi et al. [34] have shown that cell contact inhibition up-regulates LMW-PTP intra-cellular activity, and this can cause, at least in part, a reduced PDGF-R activation in these cells, showing that PDGF-R is a substrate for LMW-PTP [26]. The authors also show that LMW-PTP expression is controlled both by cell–cell contact and cell differentiation, suggesting that LMW-PTP is involved in anti-proliferative signaling pathways related to growth inhibition, and conclude that this protein behaves as a growth arrest protein [34]. In fact, one of the most relevant phenotypic effects of LMW-PTP overexpression is the strong reduction of cell

Table 1 Interaction of LMW-PTP with several molecules involved in cancer

"Cancer molecule"	LMW-PTP effects	Reference	Effect
PDGF-R	Anti-proliferative signals; growth inhibition; growth arrest protein	[26]	Anti-oncogenic
p190RhoGAP	Cytoskeleton rearrangement; increase cell adhesion, spreading, and migration	[8, 27]	Oncogenic
Ephrin A2 receptor	Increase tumorigenic and metastatic potential; dephosphorylation of EphA2	[8, 9]	Oncogenic
β-catenin	Destabilization of cell–cell adhesion	[28]	Oncogenic
FAK	Inhibition of initiation, growth, and metastasis of tumors	[29, 30]	Anti-oncogenic
JAK	Anti-apoptotic; survival pathways	[31]	Oncogenic
STAT	Inhibition of survival pathways	[32, 33]	Anti-oncogenic

PDGF-R platelet-derived growth factor receptors, *FAK* focal adhesion kinase, *JAK* janus kinase, *STAT* signal transducer and activator of transcription

growth rate in response to PDGF stimulation, together with cell adhesion and chemotaxis up-regulation [35]. Moreover, inhibition of LMW-PTP by pervanadate treatment or by the expression of a dominant negative form of the protein causes an increase in PDGF-R tyrosine phosphorylation [36].

Taken together, these data suggest that LMW-PTP can act as a physiological modulator of the effects of PDGF on cell growth, being the only PTP identified to date that is able to dephosphorylate the activation loop regulatory tyrosine of PDGF-R, thus decreasing the kinase activity of the receptor toward both the exogenous substrates and the receptor itself [37]. The interaction between LMW-PTP and PDGF-R can be important in both tumor growth and progression, although through different mechanisms [38]. In growing cells, the basal activity of PTPs is redox down-regulated by oxidation during PDGF-R signaling, which allows PDGF-R signal through downstream pathways. In differentiating cells, LMW-PTP activity is up-regulated due to ROS decrease: PDGF-R signaling is decreased, and consequently, NADPH oxidase is inactive, decreasing ROS production. This activation of LMW-PTP leads to the inhibition of PDGF-R downstream signaling, causing growth arrest [38].

Blocking growth factor receptors has long been considered a possible therapeutic target for cancer. Blocking epidermal growth factor receptor (EGFR) activity with tyrosine kinase inhibitors or monoclonal antibodies is currently used in the treatment of patients with colon cancer. Similar to EGFR, blocking PDGF-R, which will cause cancer cell growth arrest, can also be a possible therapeutic option for cancer, and LMW-PTP may be one of the molecules through which this can be achieved.

p190RhoGAP

Cell–cell adhesion, a paramount feature in oncogenesis, is regulated by multiple mechanisms, and the downstream effects of the Rho family GTPases have been recognized as a key player in this process. Rho family GTPases play a major role in directing actin dynamics and therefore have a profound impact upon these developmental processes [8]. Rho acts specifically to promote stress fiber generation [39], and Rho GTPases alternate between active (GTP-bound) and inactive (GDP-bound) conformations, binding to a multitude of downstream effector targets when in their active GTP-bound state. Their upstream regulators include GTPase-activating proteins (GAPs), guanosine nucleotide exchange factors, and RhoGTPases dissociation inhibitors [40]. One of the important GAPs is p190RhoGAP, which is involved in cytoskeleton rearrangement and seems to be one of the LMW-PTP cytoskeleton-associated fraction specific substrates [10, 27, 41]. It has been shown by Vincent et al. [42] that inactivation of RhoGAP is an effective means of promoting Rho-mediated cellular processes. Through dephosphorylation of p190RhoGAP, LMW-PTP may be involved in the regulation of the small GTPase Rho, potentiating its action, and the consequent cytoskeleton rearrangement.

This relationship between p190RhoGAP and LMW-PTP was also demonstrated in K-Ras transformed cells (Kirstein-Ras-transformed normal rat kidney fibroblast) [43]. Nox-1 generates ROS that oxidizes and inactivates LMW-PTP, resulting in the accumulation of tyrosine-phosphorylated active p190RhoGAP. Active p190RhoGAP causes down-regulation of Rho, possibly leading to the deregulation and preventing hindrance of actin stress fibers and focal adhesion assembly [43]. Deregulation of actin stress fibers may lead to changes in the adhesion and migratory ability of cells.

Given that migration is one of the hallmarks of tumor spreading, invasion, and metastization and that elevated expression levels of RhoA have been correlated with tumor stage or enhanced metastasis in several tumors, including breast cancer [44], we propose that the action of RhoA can be potentiated through LMW-PTP. Furthermore, a relationship between EphA2, a molecule that has been largely associated with tumorigenesis, p190RhoGAP, and cellular adhesion has been established through Src and LMW-PTP [8].

Ephrin A2 receptor

The Eph family of receptor kinases and their ligands, ephrins, constitute the largest receptor tyrosine kinase subfamily, with at least 16 receptors and 9 ligands [45]. This family of receptors transduces repulsion of cells in axon guidance, migration, invasiveness, and tumor growth, exerting a negative signaling effect on cell proliferation and adhesion [45]. The receptors, originally identified as modulators of axon guidance and embryonic patterning during development, were subsequently shown to be overexpressed in a large number of cancers [8].

The EphA2 receptor (EphA2) has been largely associated with tumors, and its expression levels on tumor cells correlate with the degree of tumor malignancy [46]. EphA2 is phosphorylated upon occupation by an ephrin ligand that induces a conformational change in its cytoplasmic domain triggered by phosphorylation of two juxtamembrane tyrosines, which relieves kinase domain inhibition [47, 48]. However, this mechanism is still unclear because, unlike other receptor tyrosine kinases, neither ligand binding nor receptor tyrosine phosphorylation is necessary for EphA2 tyrosine kinase activity [49].

In 2002, Kikawa et al. demonstrated that EphA2 is regulated by an associated phosphatase, identifying LMW-PTP as a critical regulator of EphA2 tyrosine phosphorylation. The overexpression of LMW-PTP in metastatic cancer cells leads to the up-regulation of EphA2. The authors conclude that LMW-PTP could be considered as an oncogene that requires EphA2 for its oncogenic activity [9].

EphA2 elevated levels are observed in some types of cancers, such as breast, prostate, and colon adenocarcinoma, as well as aggressive melanomas [45]. The fact that elevated EphA2 levels are found on multiple types of cancer suggests that EphA2 overexpression may be a common event in the metastatic progression of carcinoma cells [50]. This overexpression seems to have an important role in the regulation of adherens junctions, being important in cell–cell adhesion processes, and recent studies have reported the destabilization of adherens junctions through overexpression of EphA2 in breast tumors [8]. Fang et al. have shown that EphA2 overexpression does not affect the phosphorylation status of E-cadherin, p120, or catenins, but appears to result in the up-regulation of RhoA GTPase activity via p190RhoGAP and LMW-PTP [8]. In addition to regulating tumor cell motility, increased levels of the EphA2 receptor in tumors promote destabilization of cell–cell adhesion by regulating RhoA GTPase activity. EphA2-mediated RhoA activation is facilitated through LMW-PTP-mediated inhibition of p190RhoGAP, similar to the effects of EphA2 overexpression in mammary epithelial cells [51]. These results suggest that potentiation of EphA2 tumorigenic action seems to be dependent on RhoA GTPase [52].

Zantek et al. have shown that EphA2 in these aggressive cancer cells is not tyrosine phosphorylated [53], which suggests another possible role of LMW-PTP in the tumorigenic process through dephosphorylation of EphA2. Therefore, overexpression of EphA2 may promote destabilization of adherens junctions through a Src kinase-dependent signaling pathway, enhance LMW-PTP ACP1_001 isoform activity, inhibit p190RhoGAP, and activate RhoA GTPase.

From all the known molecules apparently regulated by LMW-PTP, EphA2 seems to be the most closely involved in the tumorigenic processes. The action of LMW-PTP on EphA2 highlights the fact that PTPs are not just suppressors of tyrosine phosphorylation-dependent signaling that are linked to a housekeeping function; they act as specific regulators of signaling pathways. Is this case, the dephosphorylation of EphA2 by LMW-PTP is closely related to its tumorigenic potential, particularly promoting alterations in the adhesive and migratory ability of the cells.

β-catenin

β-catenin plays a dual role as a major constituent of cadherin-based adherens junctions and also as a transcriptional co-activator. In normal epithelial cells, at the adherens junction level, β-catenin links cadherins to the actin cytoskeleton. The loss of β-catenin–cadherin association has been correlated with the transition from a benign tumor to an invasive metastatic cancer [28] through epithelial-mesenchymal transition. There is evidence that LMW-PTP levels increase in confluent cells compared to cells in the exponential phase, suggesting a possible role of LMW-PTP in contact growth inhibition and in the stabilization of cell–cell junctions as cells reach confluence [54]. Taddei et al. [28] have shown that the regulation of cell–cell junctions in confluent cells is due to tyrosine phosphorylation of β-catenin, and this phenomenon is influenced by LMW-PTP. The interaction between the two proteins seems to lead to a strong bond between cadherin and the actin-based cytoskeleton and could therefore play a central role in the maintenance of the architecture of solid tissues, potentially preventing tumor progression and invasiveness [28]. The interaction between LMW-PTP and β-catenin can also occur through Src inactivation by LMW-PTP, which decreases β-catenin phosphorylation and potentiates the bond between E-cadherin and β-catenin, increasing cell–cell adhesion [28].

Taken together, data from β-catenin and EphA2 suggest that the adhesiveness of cells is positively influenced by LMW-PTP, probably due to the modification of tyrosine phosphorylation levels of molecules present at the adherens junctions, hence being involved in their remodeling, a process unequivocally associated to tumor progression and metastasis.

FAK

Focal adhesion kinase is a cytoplasmic non-receptor tyrosine kinase identified as a key mediator of integrins' signaling. FAK is activated by integrins through disruption of an auto-inhibitory intra-molecular interaction between its kinase domain and the amino terminal FERM domain [55] and is implicated both in the control of cell migration, by modulating the turnover of focal adhesions, and in the transduction of cell survival signals from the extracellular matrix [29].

Rigacci et al. [29] have shown that in the NIH3T3 cell line, the main target of LMW-PTP is FAK, whose dephosphorylation culminates, via downstream events involving Src and ERK, in the observed changes of cell morphology and motility. LMW-PTP overexpression can affect FAK by two mechanisms: by causing its dephosphorylation and by impairing its adhesion-dependent redistribution to the cytoskeleton. These authors conclude that LMW-PTP dephosphorylates FAK and that cells overexpressing LMW-PTP are more motile and less spread and display a reduced number of focal adhesions, all in keeping with an increased turnover of focal contacts and a shift of the equilibrium toward their disassembly [29]. On the other hand, Src activation is crucial for many FAK actions, given that Src directly phosphorylates many tyrosine residues on FAK, thereby enhancing FAK activity and promoting the recruitment and phosphorylation of several structural and signaling intermediates [56]. Therefore, through dephosphorylation of FAK Tyr-397, LMW-PTP may prevent Src from interacting with FAK, thus interfering with the enhancement of FAK catalytic activity and the outcome of FAK-dependent biological effects [57].

Also, results by Abdelsaid and El-Remessy showed a crucial role of the expression and regulation of LMW-PTP and its importance in the activation of FAK and endothelial cell migration: endothelial cells with LMW-PTP expression silenced have a marked increase in FAK activation and cell migration [30], thus suggesting a potential role for LMW-PTP in angiogenesis. Given that angiogenesis is an essential component of the metastatic pathway, the implication of LMW-PTP in this process strengthens the role of this protein in cancer progression.

Increased levels of FAK mRNA and protein have been found in the vast majority of invasive and metastatic tumors, whereas their levels in normal tissues or benign tumors are lower [55]. A growing body of evidence suggests that FAK may be a key protein that promotes initiation, growth, and metastasis of tumors, and it has been suggested as a possible target for new therapeutic strategies. Since LMW-PTP regulates FAK activity, it too may be a putative therapeutic target.

JAK–STAT

Janus kinases are cytoplasmic tyrosine kinases that mediate signal transduction from various growth factors and cytokines [58]. The best characterized molecular event following activation of JAK kinases is the tyrosine phosphorylation and activation of a family of latent cytoplasmic transcription factors—STATs [58]. After dimerization via SH2 domains, STATs translocate into the nucleus, where they interact with specific DNA sequences to stimulate transcription [59]. The JAK/STAT pathway is pivotal in many biological processes including differentiation, proliferation, and oncogenesis [59]. In pancreatic adenocarcinoma cells, an extremely aggressive adenocarcinoma, unresponsive to chemotherapy and radiotherapy due to its resistance to apoptosis [31], it has been shown that growth factors inhibit apoptosis by activating NADPH oxidase, particularly its nox4 subunit [60]. A mechanism explaining this mediation of the anti-apoptotic effect of NADPH oxidase has been proposed: ROS produced by the NADPH oxidase complex oxidizes and inactivates LMW-PTP [31]. This inactivation, in turn, is required to enhance or maintain activation of anti-apoptotic kinases such as JAK2. It is proposed that this pathway represents a novel mechanism for the pro-survival effects of ROS and may unravel new targets for the treatment of pancreatic cancer. Therefore, ROS and the redox regulation of LMW-PTP may also be involved in cancer through JAK and STATs. In this case, cells would use LMW-PTP to proliferate and survive through ROS production, modulating its activity and ability to interact with different molecules independent of its activation or inactivation by oxidation. Therefore, even when inactivated by ROS, LMW-PTP could be beneficial for cancer cells to survive: oxidized LMW-PTP is unable to dephosphorylate and inactivate JAK2 and STAT5, increasing pro-survival signaling [4].

Rigacci et al. [32] have shown that LMW-PTP associates with and dephosphorylates murine STAT5A, interacting with its C-terminal region [32]. To better understand this process and because a mechanism capable of modulating the interaction between these two proteins may exist—otherwise STAT5 would constantly be under negative control of LMW-PTP—some authors further studied this interaction. Studies showed that cytosolic phosphatase LMW-PTP regulates the onset of STAT5 activity by associating with it and maintaining it in a tyrosine dephosphorylated state until a stimulus promotes their dissociation [33]. LMW-PTP is the only identified tyrosine phosphatase that associates with STAT5 at its C-terminus regulating its activity [32].

Cancer cells grow and proliferate more than normal cells, having STAT pathways activated. Through dephosphorylation of STAT5, LMW-PTP may block the proliferation of cancer cells, thus being important for keeping tumor cell proliferation under control.

The action of LMW-PTP in the JAK-STAT pathways seems to have contradictory roles: when inactivated by ROS, it is used by cancer cells to proliferate, while on the other hand, when activated, dephosphorylated STAT5 blocks proliferation of cancer cells. Hence, once more, cancer cells can benefit from a different activation status of LMW-PTP, strictly controlling the balance between cysteine oxidation and tyrosine phosphorylation in order to proliferate and survive [4].

Considering the above available data, we can conclude that LMW-PTP can have both oncogenic and anti-oncogenic roles, depending on the molecule with which it interacts.

LMW-PTP can act as a physiological modulator of PDGF effects on cell growth—it is the only PTP identified to date that is able to dephosphorylate PDGF-R, decreasing its kinase activity and promoting growth arrest. After the action of the slow isoform on PDGF, the fast isoform is activated and dephosphorylates p190RhoGAP, which then activates RhoA GTPase. This activation will lead to alterations in stress fiber assembly, culminating in changes in cellular adhesion and migratory ability. Also, LMW-PTP, together with Src, is one of the intermediates in the signaling pathway between EphA2 and p190RhoGAP. EphA2 confers tumorigenic potential to cells by alterations in RhoA through activation of Src, with the consequent phosphorylation and activation of LMW-PTP by Src. Regarding cellular adhesion, LMW-PTP has effects beyond p190RhoGAP and consequently RhoA GTP. It also has an important role in maintaining solid tissue architecture through β-catenin dephosphorylation, strengthening the bond between E-cadherin and β-catenin, thus increasing cell–cell adhesion. This can be important in the metastatic process at the stage of cancer cell implantation in the metastatic site.

Association of LMW-PTP and adhesion, and consequently migration, is also achieved through its activity in FAK dephosphorylation, which, via ERK and Src, leads to cellular changes and motility, decreasing the number of focal adhesions.

In addition to its importance in processes involving cellular adhesion, LMW-PTP also dephosphorylates molecules associated with survival pathways, such as JAK and STAT. LMW-PTP finely regulated activity by oxidation, and phosphorylation is used by cancer cells to promote their own survival. The interaction between LMW-PTP and JAK/STAT is a good example of how cancer cells can use this phosphatase to alter their survival potential.

Therefore, although different pathways are involved, LMW-PTP controls two cellular key points, proliferation and adhesion, which are the most important mechanisms by which cancer cells can survive and proliferate, thus rendering LMW-PTP a promising target for cancer therapy.

LMW-PTP and cancer

The physiological function of genes is one of the major challenges that will allow the understanding of the molecular basis for human disease. Human genetic polymorphisms can help identify the involvement of a given gene in human pathophysiology. There are various studies associating genetic polymorphisms of *ACP1* with different pathologies, e.g., developmental disturbances and hemolytic favism [16], systemic lupus erythematosus [61], obesity-related hypertension [62], hypertension [63], and cancer [64, 65].

We have previously reported a positive association between the fast isoform of *ACP1* and human cancers, mainly cervix and breast [64], but Spina et al. [65] have shown that the protective effect of the slow isoform in colon cancer patients prevails over the predisposition effect of the fast isoform. The apparent disparity between these studies may be related to the type of cancer and tumor stage. It is presently unknown if the differential expression of the two isoforms due to the ACP1 polymorphism may have different roles in tumorigenesis and cancer progression or if different types of cancer cells express different levels of the LMW-PTP isoforms. There is a clear need to evaluate different stages of tumor progression and metastization in order to understand the relationship between these polymorphisms and the pathophysiology of the disease, and in vitro studies have been conducted in order to better understand the underlying mechanisms. Chiarugi et al. [7] have shown that while LMW-PTP negatively regulates growth factor-mediated proliferation in NIH3T3 mouse embryonic fibroblast cultures, in animal models, it acts as a positive regulator of tumor onset and growth [7]. These authors also showed that despite the fact that LMW-PTP overexpressing cells are endowed with enhanced in vitro adhesion and mobility, their engrafts did not lead to metastasis. Malentacchi et al. [66] evaluated the expression levels of LMW-PTP mRNA in different human carcinomas—breast, colon, lung, and a group of neuroblastoma samples as representative of a neuroendocrine cancer. Results strongly suggested a common pattern, by which an increase of LMW-PTP expression, irrespective of the isoform, is observed in most tumor samples, with the exception of lung cancers. LMW-PTP protein content was in agreement with the observed increase in mRNA, confirming that the overexpression of LMW-PTP mRNA leads to LMW-PTP protein overproduction [66]. Based on these results, the authors concluded that the *ACP1* gene can be considered an oncogene.

This overexpression of LMW-PTP in different types of cancer is consistently found in tumor cells that have high levels of unphosphorylated EphA2. LMW-PTP might regulate EphA2 expression, function, or both, and indeed, ectopic expression of LMW-PTP causes dephosphorylation and up-regulation of EphA2 [9, 49].

Figure 2 schematically represents the various interactions of LMW-PTP isoforms with different molecules and signaling

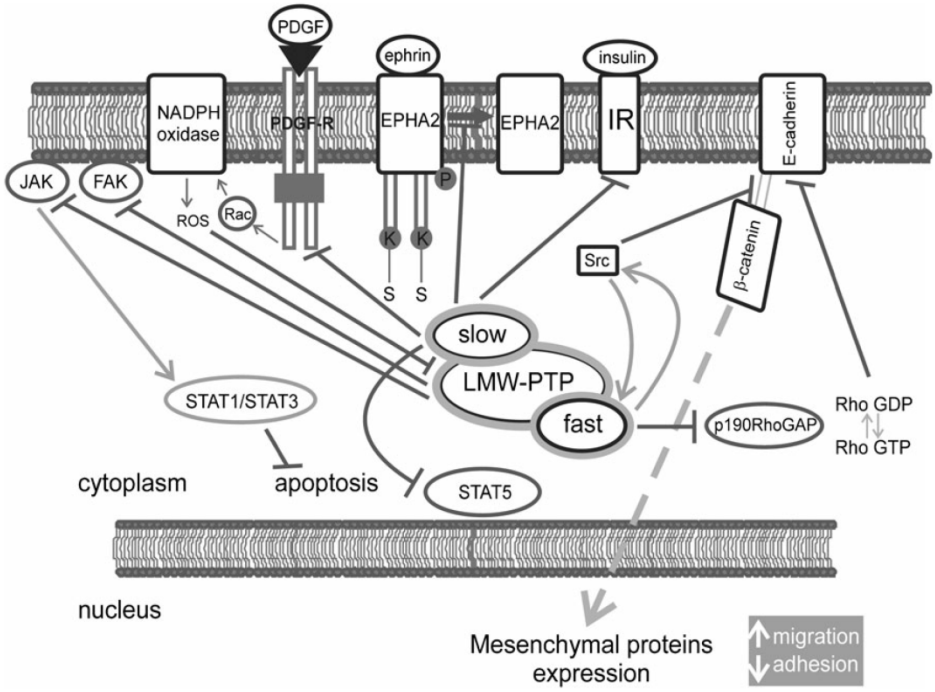

Fig. 2 Interactions between LMW-PTP and cancer-associated molecules. LMW-PTP inhibits important molecules that may be involved in cancer processes. LMW-PTP inhibits FAK, thus decreasing the number of focal adhesions and cell migration; it also inhibits JAK, thus stimulating STAT1/STAT3 and promoting survival pathways via inhibition of apoptosis. LMW-PTP slow isoform is able to interact with STAT5, inhibiting its activation and translocation to the nucleus. LMW-PTP slow isoform prevents PDGF signaling, promoting growth arrest. On the other hand, upon PDGF binding to PDGF-R, there is an increase of ROS production through activated NADPH oxidase via Rac, which causes LMW-PTP inhibition. Dephosphorylation of EPHA2 by LMW-PTP slow isoform increases tumorigenesis and metastatic potential, promoting transformation, and IR dephosphorylation decreases glicogenosynthesis rate affecting mitogenesis. The fast isoform dephosphorylates and inhibits p190RhoGAP and consequently potentiates Rho action which, through E-cadherin, destabilizes adherens junctions. An important molecule in this process is Src: Src can be activated by ROS, and activated Src associates with the fast isoform of LMW-PTP. This association induces tyrosine phosphorylation and activation of LMW-PTP, which, in turn, promotes Src activation. Src is able to inhibit the association between E-cadherin and β-catenin, promoting β-catenin signaling action and consequently increasing the expression of mesenchymal proteins, enhancing migration and decreasing cell adhesion

pathways. Contradicting these evidences, recent studies [67] showed that suppression of some PTPs, such as LMW-PTP, enhanced migration of mammary epithelial cells, supporting an anti-oncogenic role for LMW-PTP.

Two recent studies address the role of LMW-PTP in cancer treatment: one is based on the chemoresistance of cancer cells and the potential of LMW-PTP to be involved in this mechanism [68]; the other considered LMW-PTP as an emerging target for the design of novel therapeutic agents [69]. Multidrug resistance development limits the efficacy of continuous treatment in different types of tumors. Ferreira et al. showed that LMW-PTP is involved in chemotherapy resistance in leukemia through the maintenance of both Src and Bcr-Abl kinases in a more active status [68]. Moreover, Maccari and Ottaná highlight the importance of developing new compounds that are isoform specific, given that selective inhibitors of LMW-PTP isoforms are not only considered of medical interest but can also be useful to further investigate the molecules, pathways, and cellular mechanisms in which these proteins are involved, both in physiological and pathological processes [69].

The relationship between LMW-PTP and oncogenesis remains controversial: different studies have reported different and sometimes opposite results and conclusions. We suggest that one possible explanation for these apparently contradicting results lies in the different effects of the two main isoforms in tumorigenesis and in the different stages of tumor progression. The analysis of different types of tumors in different stages would help clarify this relationship and explain the apparently controversial role of LMW-PTP in the tumorigenic process. Further studies are being conducted in order to confirm our hypothesis and open new avenues of research regarding the importance of LMW-PTP isoforms in cancer.

Conclusions and future directions

The effects of LMW-PTP on tumorigenesis and cancer progression are clearly controversial. There is a need to address if LMW-PTP isoforms are associated with tumorigenesis or tumor progression. The relative importance of the two isoforms may depend on tumor type or stage.

The importance of genetic polymorphisms, their relationship with the isoforms, and, consequently, different enzymatic activity at tumor onset, growth, progression, and metastization are currently extremely important issues that need further research.

As suggested throughout this review, Src may be a key intermediate between LMW-PTP and the molecules it regulates, thus being involved in LMW-PTP potential action in tumorigenesis. Given that Src inhibitors are currently being used in cancer research, LMW-PTP may be an emerging novel target molecule for cancer therapy.

Therefore, we propose that studies focusing on the differential roles of LMW-PTP isoforms should be the aim of future research in order to ascertain if the two major isoforms may be possible novel prognostic markers or therapeutic targets.

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Conflicts of interest None.

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Chapter 8

Global discussion and conclusions

8 Global discussion and conclusions

The results obtained in the course of the present work have provided major insights into the function of LMW-PTP and its isoforms in cellular processes that are important for cancer cells to spread and metastasize, namely to bone. These findings are discussed in each of the papers presented in previous chapters, and will be interpreted, in this chapter, in a unifying manner and in light of cancer translational research, based on tumor-host interactions.

Translational research is an important approach to apply the knowledge of basic research to clinical practice. During the development of this work we always kept in mind that our findings and results obtained in the bench could help to explain some facts with which medicine deals every day.

Bone metastases are an excellent example of how the tumor needs the host and its microenvironment. Bone tissue is a specialized connective tissue rich in growth factors and cytokines, providing an excellent *soil* for tumor cells to grow. Tumor cells growing in bone are therefore "*parasites*" that could grow in a different microenvironment, being dependent on it, using factors released during bone resorption to survive and grow, causing damage to the host, namely unbalancing the bone remodeling process. These facts are the rationale for the current use of two types of therapies in patients with bone metastases: targeting tumor cells and targeting bone cells, namely osteoclasts (1). Throughout this work, we aimed at clarifying how LMW-PTP and its isoforms could be important in the bone metastization process and in therapeutic response. In the context of bone metastasis, the use of bisphosphonates in patients with metastatic bone disease provides a normalization of NTX levels in around 75% of the patients. The reason why the other 25% do not respond to therapy is not clear. We hypothesized that it can be due, at least partially, to the genetic variability of patients, namely variability of the osteoclasts. LMW-PTP, a widely expressed polymorphic enzyme, could contribute to this genetic variability and consequently to the differential therapeutic response in patients with bone metastasis under bisphosphonates therapy.

LMW-PTP and its isoforms were the target of our study due to the association of this protein with bone metabolism, namely osteoblastic differentiation (2, 3), due to its association with cancer (4) and due to our preliminary results regarding the association of LMW-PTP isoforms with cancer (5).

Although these different studies were performed on an attempt to clarify the importance of LMW-PTP in cancer, there are no functional studies associating separately the two main isoforms with cancer and their correlation with therapeutic response

There is a paucity of information regarding the different functions of the two main LMW-PTP isoforms in the cell. It is known that the *slow* isoform, in the cytosolic pool, binds to and dephosphorylates activated PDGF-R, whereas the f*ast* isoform, in the cytoskeletal associated pool, specifically acts on substrates such as p190RhoGAP, which become tyrosine phosphorylated upon PDGF treatment. These observations demonstrate that upon PDGF stimulus, the two constitutive LMW-PTP pools possess marked differences in enzyme-specific activity that could reflect different roles in signal transduction (6).

Since it seems that LMW-PTP controls cellular vital functions through the interaction with PDGF-R and p190RhoGAP, the role of this phosphatase in oncogenesis may be crucial. However, and although different studies have been published concerning the role of LMW-PTP in oncogenesis (4, 5, 7-9), its role remains controversial. Studies showing the relevance of the two main isoforms are lacking, remaining the importance of these proteins unclear. Therefore, we explored the role of LMW-PTP in tumor progression, deepening LMW-PTP isoforms functional studies, in an attempt to clarify if the two main isoforms have different roles during this process.

The association between LMW-PTP and cancer lies in different supportive data: LMW-PTP polymorphism has been associated with predisposition for some type of cancers (5, 9); LMW-PTP is overexpressed in tumor tissue compared with normal tissue (4); LMW-PTP interacts with different molecules strongly associated with cancer such as EhpA2 (8, 10) , RhoA (10), and Src (11), being consequently implicated in important cellular features such as adhesion and migration.

Although our main goal was to understand how LMW-PTP isoforms can be important for bone metastasis, due to the lack of studies regarding LMW-PTP isoforms in cancer, in general, our functional studies started with the characterization of a panel of breast cancer cell lines regarding LMW-PTP isoforms expression compared with a normal breast cell line (Chapter 2). On this primary evaluation we observed a decrease of the *fast* isoform expression in all cell lines and an increase of the *slow* isoform in almost all cell lines compared to the control. This was our first evidence that the expression of LMW-PTP isoforms is changed in tumors compared to normal tissue. This study was

the basis for the choice of MDA-MB-435 as the cell line to be used in subsequent studies. The selection of this cell line was due to its high invasive potential and because it was the cell line with the highest expression of the *slow* isoform and the lowest expression of the *fast* isoform. Attempting to overexpress the two LMW-PTP isoforms was our first approach in order to perform functional studies. Unfortunately, we were not able to achieve a stable overexpression of these proteins, which was required to proceed with further studies. After 48h of transfection the levels of LMW-PTP and its isoforms' expression returned to basal levels. Only 24h after transfection we observed an increase of LMW-PTP and its isoforms' expression levels (Results in appendix 1)

Given these results, we hypothesized that in the MDA-MB-435 cell line LMW-PTP expression is finely regulated and overexpression of these proteins for more than 24h will lead to cell death. Thus, these cells apparently have an intrinsic mechanism that downregulates LMW-PTP levels in order to survive.

To further study the functional role of LMW-PTP and its isoforms, we tried to downregulate LMW-PTP expression using siRNAs inserted in lentivirus.

The available sequences of siRNA only target LMW-PTP total and its *slow* isoform. After exhaustive post-infection clone analysis, by mRNA expression and enzymatic activity, we chose one clone that showed knockdown of the *slow* isoform and one clone that showed a more effective knockdown of total LMW-PTP.

Using the selected clones we performed some functional assays that would enable us to explore the role of the two isoforms in the context of human tumors. First of all we evaluated the proliferation rate of the clones. Proliferation is one of the main characteristics of tumors cells, namely primary tumors. Based on our preliminary studies showing an increase of LMW-PTP *slow* isoform in breast tumors and the association of this isoform with PDGF-R (12) and EphA2 (8), we expected an increase in proliferation in the selected clones. However, our results showed that the selected clones had a growth rate comparable to controls. We hypothesize that this may be due to at least two different mechanisms: 1) the high proliferation rate of this cell line, which has a population doubling time around 22h, may hinder a further increase on cell proliferation rate, which would cause the cells to die due to important metabolic alterations that would contribute to the cells to become unviable; 2) phosphorylation status of EphA2.

EphA2 appeared overexpressed in different types of tumor and its overexpression is accompanied by LMW-PTP overexpression conducting to dephosphorylation of EphA2 (7). Unphosphorylated EphA2 has been associated with the increase of tumor growth and transformation of tumor cells (13). Some authors refer that the oncogenic potential of EphA2 is dependent of LMW-PTP once this phosphatase is able to dephosphorylate EphA2 (8). However in our model we did not observe this relationship. Also, Fang et al demonstrated that in MCF10A cells, LMW-PTP is not able to interact with EphA2 (10), which is in accordance with our results. Therefore, and given we did not see any difference in the phosphorylation status of EphA2 in the LMW-PTP KD cells, the absence of differences in the proliferation rate between the selected clones and control could be due to the unaltered phosphorylation of EphA2.

The pathway that we proposed to study during this work was the axis EphA2- LMW-PTP – p190RhoGAP – RhoA, and consequently migration and invasion potential of the cells. We considered LMW-PTP as a central protein in this pathway, and tried to explore how the differential expression of the two isoforms can be relevant for the interpretation of the results in a context of tumor behavior and and cancer progression.

After the proliferation assay, migration and invasion assays were performed. In the migration assay, suppression of LMW-PTP caused an increase of cellular migratory potential. Recently, Lin G et al, using RNAi mediated loss-of-function screen of PTPs, demonstrated that suppressing a group of PTPs where LMW-PTP is included, increased the migratory potential of the cells (14).

Also, Chiarugi et al (7) showed that in NIH3T3 cells, the overexpression of wtLMW-PTP leads to a strong increase in cell motility showing that LMW-PTP has a positive role in the regulation of cell motility, even through a physical barrier of reconstructed lamina or through rapid colonization of the wound. In agreement with this, the expression of MMPs, namely MMP8, MMP9 and MMP13, is increased in these cells (7).

These authors also showed that the expression of LMW-PTP dominant negative had the opposite role. This fact seems to be in contradiction with our results showing that the suppression of LMW-PTP increase the motility of the cells. The studies were performed with different cell lines – we used a breast cancer cell line (MDA-MB-435) whilst others authors used murine fibroblasts (NIH3T3) - demonstrating that the two cell lines show different behaviors when LMW-PTP expression is altered. Given our results we

hypothesized that the increase in the migratory potential of the cells when LMW-PTP is suppressed could be due to the interaction with p190RhoGAP and consequently RhoA. Our results showed that LMW-PTP could change RhoA activation status: whilst KD LMW-PTP *slow* isoform increases RhoA activation, the knockdown of the total protein decreases RhoA activity. Only the *fast* isoform has been described as a regulatory protein of RhoA, through dephosphorylation of p190RhoGAP. However, our results demonstrated that the two main isoforms of LMW-PTP can regulate RhoA activity, although in opposite ways: the *slow* isoform seemed to inactivate and the *fast* isoform seemed to activate RhoA. Irrespective of the effect in RhoA, the final effect on migratory potential is the same - cells were able to migrate more than controls.

We speculate that if we can achieve an overexpression of LMW-PTP and its isoforms, the result in the migratory potential would be the same. The rationale for this speculation is that the balance between RhoAGTP/RhoAGDP is a crucial mechanism that controls the migratory potential of cells. As it is known, RhoGTPases are a family of proteins that play a pivotal role in the biochemical pathways that are most relevant to cell migration(15). Rho is thought to regulate the contraction and retraction forces required in the cell body and at the rear. Rho activity in the front of a migrating cell is incompatible with membrane protrusion and hence mechanisms must be placed to inhibit its activity at the leading edge (15). This confirms that the alternation between conformation RhoA GTP and RhoA GDP is important for the control of cellular migration, thus supporting our speculation.

Although the suppression of LMW-PTP increases the migratory potential of MDA-MB-435, the invasive potential of these cells was not altered. Based on the results from Lin et al (14) in PTPN23, we hypothesized that this result could be due to the interaction between LMW-PTP and Src. These authors demonstrated that in the absence PTPN23, Src becomes activated, increasing the phosphorylation of E-cadherin, the release and activation of β-catenin and the internalization of E-cadherin. The regulation of E-cadherin and β-catenin phosphorylation disrupts their association and promote β-catenin activation, contributing to an increased expression of mesenchymal proteins that promote mammary epithelial cell motility, scattering and invasion (14).

We demonstrated that Src was more inactive in the KD LMW-PTP *slow*. Src is an important protein that contributes to the maintenance of normal cell homeostasis and control cellular physiological functions including cell proliferation and survival,

regulation of cytoskeleton, adhesion, migration and invasion(16). Different reports associate LMW-PTP and Src and it is known that exists a regulatory loop between LMW-PTP and Src, being these two proteins mutually regulated (3, 17).

As previously described, Lin and colleagues (14) justified the increase of the invasive potential when PTPN23 is suppressed by Src activation and the consequent increase of mesenchymal proteins expression, a characteristic of the invasive cells. The inactivation of Src in the cells with LMW-PTP *slow* isoform knockdown can explain the absence of difference in the invasive potential of the cells probably through the pathway that involves E-cadherin and β-catenin. Regarding the important relation between E-cadherin and β-catenin, both Src and LMW-PTP can have a preponderant role, as explained in Figure 1.4 in Chapter 1 (Introduction). The inactivation of the two proteins, LMW-PTP and Src, could justify the lack of differences in the invasive potential of the clones.

Regarding the relation between the two LMW-PTP main isoforms and Src, it has been reported that Src only phosphorylates the cytoskeletal pool of LMW-PTP – *fast* isoform (6). However we found that only when the *slow* isoform is suppressed, Src is more inactive. Thus, we suggest that the feedback loop between LMW-PTP and Src is dependent on the two isoforms in a distinct manner. Even if only the *fast* isoform became tyrosine phosphorylated by Src, the control of Src activation is done by the two LMW-PTP isoforms, probably in opposite ways: the two isoforms can have distinct affinities for Src Tyr 416 and Src Tyr 527. In chapter4we show that in the KD LMW-PTP *slow* increases phosphorylated Tyr 527 and also Try 416 non-phosphorylated, indicating that *slow* isoform has more affinity towards Tyr 527. In the KD LMW-PTP total we did not see any differences in the activation status of Src, again suggesting that the *fast* isoform was compensating the action of the *slow* one in the regulation of Src activity.

As described in the Chapter 1(Introduction) in vitro, bisphosphonates also have anti-tumor properties. Therefore, to conclude the studies in tumor cell lines, we evaluated the sensitivity of MDA-MB-435 to bisphosphonates when LMW-PTP was suppressed. We showed that LMW-PTP KDs did not affect the sensitivity of MDA-MB-435 to bisphosphonates (Appendix2).

After these functional assays where we discussed the possible roles of LMW-PTP isoforms in crucial cellular processes for tumor behavior , we focused our studies in the

role of LMW-PTP in bone metastization, namely in the regulation of osteoclastic activity. As previously described, tumor cells seeded in bone and bone cells drive a vicious cycle where the factors released by each cell type stimulate other cell types. (18).

We evaluated how the suppression of LMW-PTP in MDA-MB-435 cell line could interfere with the capacity for RAW 264.7, a mouse monocyte cell line, to differentiate into osteoclasts. This cell line, in the presence of RANKL, differentiates into giant, multinucleated, TRAP positive cells – osteoclasts. Our results showed that, in the presence of RANKL, factors released from the MDA-MB-435 cell line did not have the capacity to increase RAW 264.7 osteoclastogenesis compared to the positive control. Although some authors described that MDA-MB-435 conditioned medium influences osteoclastic differentiation (19), there are contradictory results (19-22). These contradictory results could be explained by the different clones that are used to perform the experiments. Moreover, our results showed that factors released from cells with the *slow* isoform suppressed decreased osteoclastogenesis of RAW 264.7 cells. Thus, we suggest that, in tumor cells, the *slow* isoform may have a prominent role in the osteoclastogenesis.

As already described, tumor released factors stimulate osteoblasts to secrete RANKL, which in turn will stimulate osteoclasts. However, osteoclasts are also directly stimulated by tumor released factors. In our experimental approach osteoblasts were not present, so the observed differences in osteoclastogenesis were due to the direct communication between tumor cells and osteoclasts.

A large number of factors released by tumor cells can induce osteoclastogenesis. We showed that Src is less active when LMW-PTP *slow* isoform was suppressed in MDA-MB-435. The less active Src was confirmed by Western Blot analysis of phosphorylated 416 and non-phosphorylated Tyr 527, demonstrating an increase in Tyr 527 phosphorylated and also Tyr 416 non-phosphorylated (Chapter 4).

Src is a crucial molecule for the function of osteoclasts (23-25) because when Src is suppressed these cells fail to resorb bone once they do not form the ruffled border (26). Src expression in osteoclasts is particularly high and it is activated in the process of RANK signaling as well as following integrin binding during bone resorption (24). However, the role of Src in the crosstalk between tumor cells and bone cells has not been described. Zhang et al (27) reported that Src supports breast cancer cell survival in

the bone marrow microenvironment by facilitating CXCL12-CXCR4-AKT signaling and by conferring resistance to TRAIL. The Src-dependent signaling and the metastatic cell survival could provide mechanistic insights into metastasis latency (27). Src has just been recently associated with cancer invasion having a critical role in organizing invadopodia. Invadopodia are subcellular protrusions found in invasive cells that posses extracellular matrix degrading activity (28) through MMPs. ROS production by Nox enzymes promotes the formation of invadopodia and activates Src, what leads to MMPs secretion (16). Overexpression of MMPs, namely MMP1, in a large panel of breast cancer is associated with increased risk of bone metastases (29). The specific involvement and functional mechanisms of individual metalloproteinases in bone metastasis remain poorly characterized.

Inactivation of Src in tumor cell lines could implicate a decrease in MMPs expression, As described in the Chapter 1 (Introduction), the involvement of MMPs in bone metastases has largely been described. A broad spectrum of MMP inhibitors have been tested in preclinical models of bone metastases and proven to be effective in bone destruction. However, the specific role of each MMPs in bone metastasis remains unclear (30). Also, the relationship between LMW-PTP and MMPs was described in NIH3T3: overexpression of LMW-PTP increased the production of MMPs (7).

Inhibition of Src and MMPs seems to be important approaches for the treatment of bone metastases. Several potential compounds have been developed and preclinical studies with these small-molecule Src inhibitors have been shown efficacy in reducing bone metastasis progression (25). Currently, different Src inhibitors such as Saracatinib and Dasatinib are being evaluated in clinical trials (31). Regarding MMPs inhibitors, Yoneda et al demonstrated that overexpression of TIMP-2, which is a natural inhibitor of MMPs are also known to play a crucial role in several common steps of cancer metastases into a human estrogen-independent breast cancer cell line MDA-MB-231, decreasing osteolytic bone lesions and increasing the survival rate of tumor-bearing nude mice (32).

Finally we studied two factors released by tumor cells that are known to be direct modulators of osteoclastic activity: IL8 and IL6. Bendre et al described IL8 as a potent direct activator of osteoclastic differentiation independently of RANKL, involving the IL8 receptor (CXCR1) on the surface of osteoclasts and their precursors (20, 33). IL8 directly supports the maturation of bone metastases in model systems (34).

Our results showed that MDA-MB-435 with LMW-PTP suppressed secreted lower levels of IL8 to the medium compared to controls. Thus, LMW-PTP could interfere with the production of IL-8 in these tumor cells. Therefore, we hypothesized that the decreased osteoclastogenesis in RAW 264.7 cells exposed to conditioned medium from MDA-MB-435 with the LMW-PTP *slow* isoform KD could be partially due to the decrease of IL-8 production.

The fact that only *slow* isoform suppression decreased osteoclastogenesis lead us to conclude two aspects: 1) the *slow* isoform has the ability to change the secretion of IL-8 but 2) other factors involved in osteoclasts differentiation are being changed by the *fast* isoform. This would explain the fact that, although the secretion of IL-8 is decreased in the two knockdowns, only the conditioned medium of MDA-MB-435 with the *slow* isoform suppressed decreased the differentiation of osteoclasts.

IL-6 is less expressed in MDA-MB-435 than IL8 (19) and some authors reported that MDA-MB-435 do not produce IL-6. (35). Our results showed that although the concentration of IL-6 in the supernatant of MDA-MB-435 was less than IL-8, this cytokine was also secreted by this breast cancer cell line, but was not dependent on LMW-PTP expression. These results suggest that IL-6 is not implicated in the process of osteoclast differentiation, which could be dependent on LMW-PTP expression in MDA-MB-435 tumor cells.

Summarizing this part of the work, we conclude that the two isoforms seem to be involved in different ways in tumor behavior and progression: the two isoforms have different levels of mRNA expression in a panel of breast cancer cell lines, and can contribute to the migration of MDA-MB-435 cells. Specifically in the interaction between tumor cells and bone cells, LMW-PTP *slow* isoform seems to have the preponderant role, having the capacity to alter the communication between breast cancer cells and osteoclasts.

To further clarify the importance of LMW-PTP in the different stages of tumor progression, we analyzed mRNA expression of LMW-PTP and its isoforms in breast tissue in different stages of tumor progression: normal breast tissue, primary tumor breast tissue and bone metastatic breast tissue. In Chapter 5 we showed that during tumor progression the expression of total LMW-PTP and its two main isoforms was changing, namely the total LMW-PTP, which is increased in primary breast cancer tissue, and the *fast* isoform, which is increased in metastatic cancer tissue. These results

seem to be contrary to the results discussed in Chapter 4: 1 - the absence of differences in Src activation (that we attribute to the action of the *fast* isoform) levels in cells with the LMW-PTP total knockdown; 2- the decrease of osteoclastogenesis in cells exposed to the conditioned medium from tumor cells with the *slow* isoform knockeddown. During this work, we have tried to explore different stages of tumor progression, but we are not able to study the bone microenvironment. Given in our *ex-vivo* studies the slow isoform seems to be more associated with osteoclastogenesis, the increase of the *fast* isoform expression in the bone metastatic breast tissue may be a consequence of all the interactions between tumor cells and bone cells that happen in the vicious cycle of bone metastasis. These results strengthen our conclusion that during the different stages of tumor progression, LMW-PTP and its isoforms could have different functions. However, further studies are necessary to explore and confirm these different roles.

Although the association of ACP1 polymorphisms and cancer has been reported (5, 9), the correlation of ACP1 polymorphisms with therapeutic response and prognosis has not been addressed. In order to evaluate if LMW-PTP isoforms could interfere in the response to bisphosphonates therapy in bone metastasis patients, we correlated urine NTX levels with ACP1 genotypes (Chapter 6), in an attempt to correlate the response to therapy based on the host's ACP1 genotypes. During bisphosphonates therapy, the levels of urinary NTX were measured monthly, as marker of bone resorption and, consequently, as a marker of bisphosphonates therapy effectiveness. Since around 25% of patients under bisphosphonates therapy are non responders (36, 37), our goal was to understand if LMW-PTP isoforms could contribute to this differential response.

Our results showed that patients' response to bisphosphonates therapy was not dependent on ACP1 genotype. Also, ACP1 genotypes did not correlate with different prognostic markers, such as SREs or time to bone disease progression. Moreover, overall survival was not affected by ACP1 genotypes. Thus, we conclude that although the two isoforms seem to be differentially associated with the different stages of tumor progression, the two isoforms cannot be considered a marker of therapeutic response or a prognostic marker. This could mean that, although the LMW-PTP isoforms could be important in tumor progression, the response to therapy is not dependent on the host's ACP1 genotypes.

Taken together our results suggest that LMW-PTP isoforms are differentially involved in the process of tumor progression, with the slow isoform apparently being more

independent of the tumor microenvironment than the fast isoform. In an early stage of tumor progression, the fast isoform expression is decreased, but in metastatic tissue it is increased. Thus, we hypothesize that, in metastatic tissue, where the bone vicious cycle is well established, with signaling between tumor and host cells being accomplished by paracrine factors such as cytokines, chemokines and growth factors (38), and the tumor is thriving, the fast isoform is being regulated by the microenvironment and this microenvironment has the ability to increase the fast isoform expression possibly as an enhancement mechanism in response to the osteoclastogenic potential of the slow isoform. Figure 8.1 summarizes and integrates our results in light of our hypothesis.

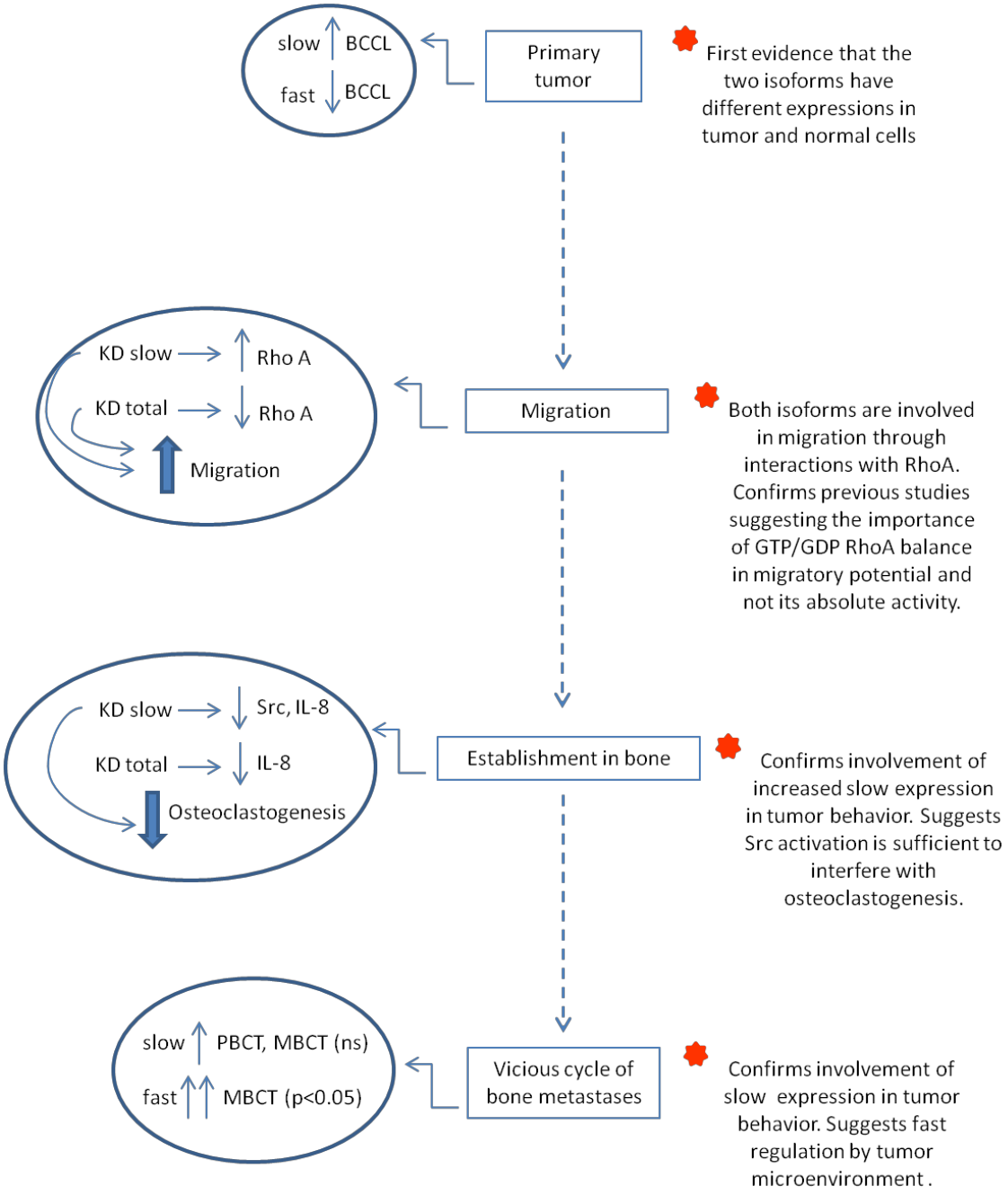

Figure 8.1- Summary and integration of our results.

8.1 Concluding Remarks

Based on our results, we conclude that the controversial role of LMW-PTP in oncogenesis is due to the paucity of information regarding the two isoforms. Our results show that the two isoforms may have different roles in the different stages of the oncogenic process: in an initial phase, the *slow* isoform may have a preponderant role, as an important molecule controlling cellular proliferation. During tumor cell migration and spreading the two isoforms seem to be involved through the regulation of RhoA and, in a final stage, in tumor metastases, the *fast* isoform seems to have a notable role, possibly in response to the vicious cycle of bone metastases.

The differential functions of LMW-PTP isoforms in the different stages of oncogenesis highlights the possibility of these proteins to be considered therapeutic targets: the *slow* isoform could be a prognostic marker for tumor progression and a therapeutic target in this early phase. LMW-PTP as a whole would be relevant as a therapeutic target in the cell migration stage, and the *fast* isoform in the final metastatic stage. Finally, host´s ACP1 genotype could not be used as a therapeutic response marker or prognostic marker in bone metastasis patients.

These results strongly suggest that LMW-PTP and its isoforms should be considered independent of each other and not as a simple “whole as the sum of its parts”. Furthermore, future studies should focus on each of these isoforms, exploring their *in vivo* role in the context of tumor progression.

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Appendix

9 Appendix

9.1 Appendix 1

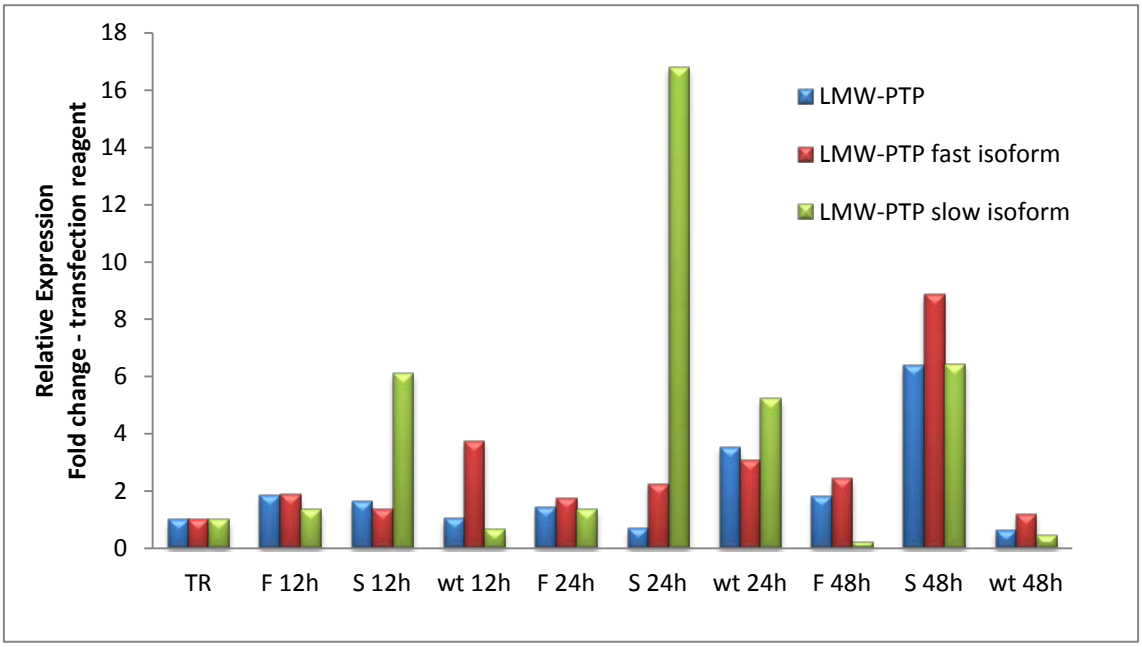

Figure 9.1 - LMW-PTP, LMW-PTP *fast* isoform and LMW-PTP *slow* isoform expression in MDA-MB-435 cell line 12h, 24h and 48h after transfection with three different plasmids containing specific sequences for total LMW-PTP (wt), *fast* isoform (F) and *slow* isoform (S), compared with cells exposed to the same time to transfection reagent (TR).

9.2 Appendix 2

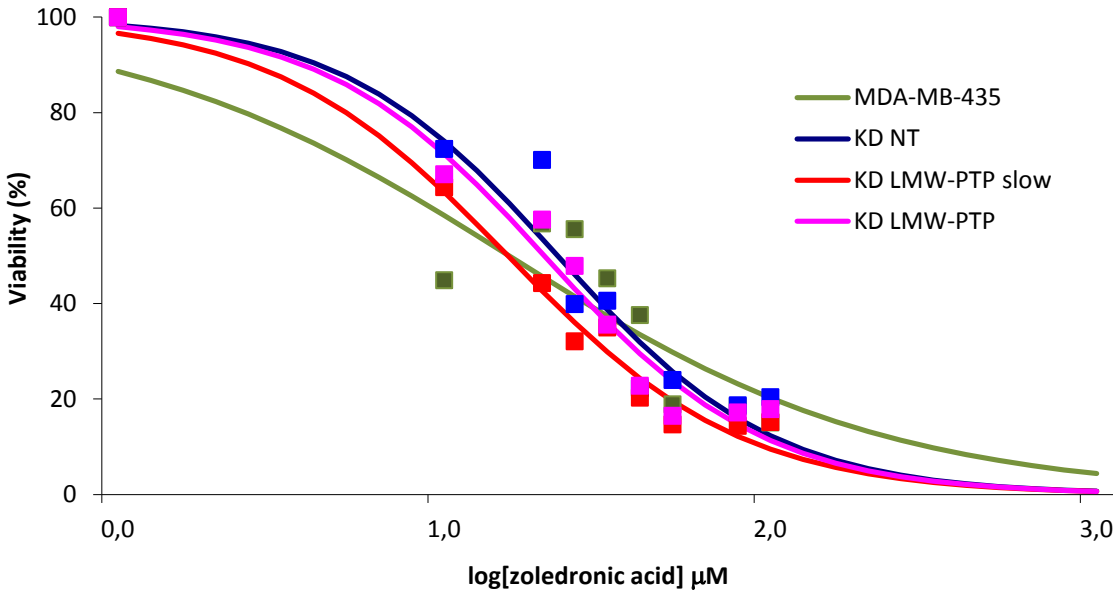

Figure 9.2- Toxicity assay of MDA-MB-435 and clones with LMW-PTP knockdown to zoledronic acid.